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DE93 003146

RESEARCH ACCOMPLISHMENTS DURING GRANT PERIOD

Introduction

Rh-105 and Au-199 have nuclear properties suitable for both therapy and imaging (moderate energy β, γ for imaging, and reasonable reactor produced $t_{1/2}$). The objectives of the project have been to develop bifunctional chelate ligands which can be used with metal ions such as Rh(III) and Au(III), to show that these Rh(III) and Au(III) complexes can be conjugated to protein molecules such as antibodies, to examine assay methods for coupling efficiency and estimation of immunoreactivity, and to test complexation of some of our ligands with other potentially useful radioisotopes for imaging and therapy. In addition we will do *in vivo* animal biodistribution studies.

In the following sections a summary of our more definitive studies is included.

Work Completed (1986-89)

During the original grant period we established (1) a procedure for production of Rh-105 at MURR (Missouri University Research Reactor). This procedure currently allows the production of 100 mCi of Rh-105 weekly. Targets of ruthenium metal (~99% enriched in Ru-104) are irradiated in a thermal neutron flux of ~8×10^{13} neutrons/cm^2·s for periods of three days or longer

$$^{104}Ru + n \text{ -----> } ^{105}Ru \text{ -----> } ^{105}Rh + \beta^-$$

$$\sigma = 0.5b \qquad t_{1/2} = 4.4 \text{ hr} \qquad t_{1/2} = 35.5 \text{ hr}$$

Details of the separation and purification were developed and now produce high quality Rh-105.

Several new bifunctional chelating ligand systems were developed as shown in Figure 1.

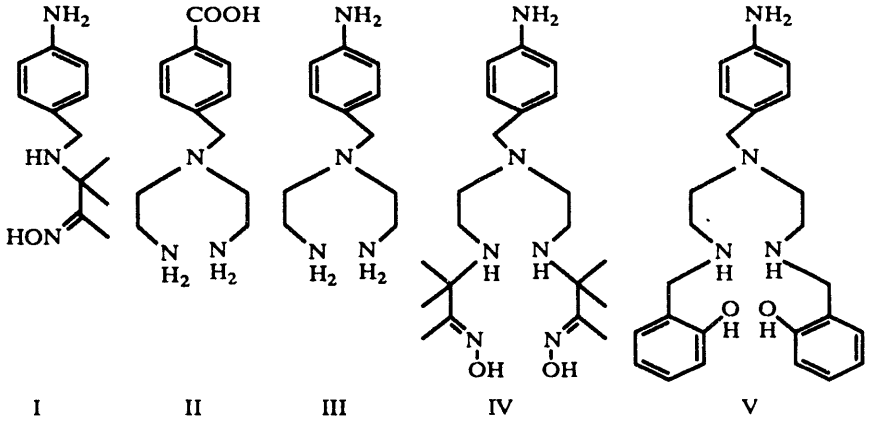

Fig. 1 Ligands found to be useful for preparing bifunctional chelates of Rh-105.

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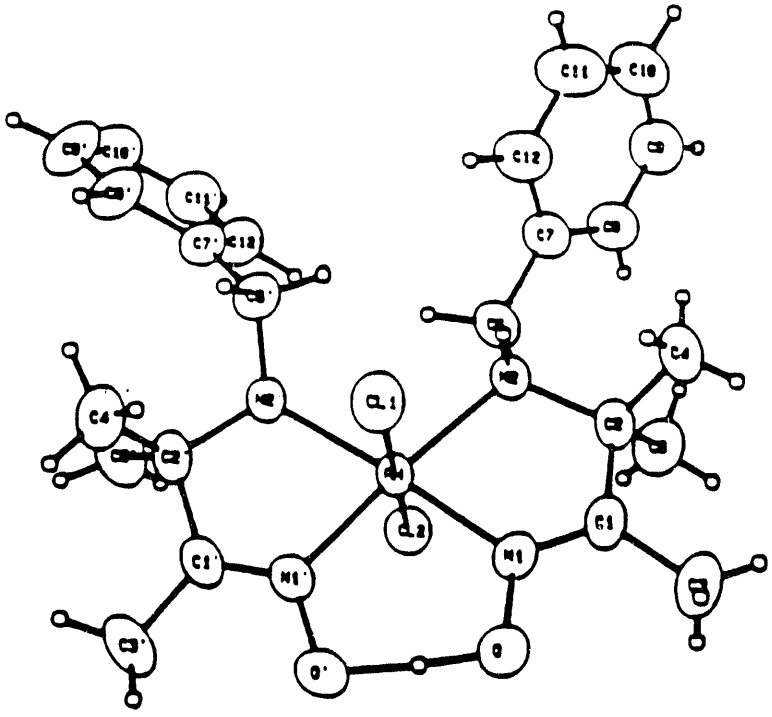
These were selected as potential ligands for Rh(III). In addition a number of the simple benzyl analogs were prepared in preliminary studies, and the x-ray structural studies (Figure 2) of the Rh(III) complexes of these analogs of ligands I and III suggested that the NH_2 or COOH functionalized counterparts would have the functional group readily accessible for potential binding to a protein (antibody).

Indeed results of antibody coupling as shown in Table 1 indicate very successful linkage to IgG. Ligand II was activated with isobutylchloroformate while all others were activated with thiophosgene. The yields are coupling yields and do not reflect losses during complexation and activation.

Table 1. Summary of Yields of Coupling Bifunctional Chelates to IgG

Ligand	~Rh/IgG Ratio	Incub. Time (hr)	Coupling Yield (%)	~Rh atoms/ IgG
I	0.5	4	76	0.4
	0.9		90	0.8
	2.3		87	2
II	1	24	38	0.4
	2		38	0.8
	5		33	1.7
III	1	4	75	0.8
	2		66	1.3
	5		56	2.8
V	2.0	4	87	1.7
	0.84		92	0.8
	0.38		90	0.4
	1.4		86	1.2
	0.88		91	0.8

A typical elution profile of the labeled IgG on a G-75 Sephadex gel permeation column is shown in Figure 3 for the complex with ligand V. Ligand V gives good yield with Rh(III) and has shown versatility in application (Table 2) to other metal ions (Tc-99m, Cu-64, Cu-67, Pd-109). The Cu(II) complexes are labile, but the others appear to be sufficiently inert for possible *in vivo* applications.



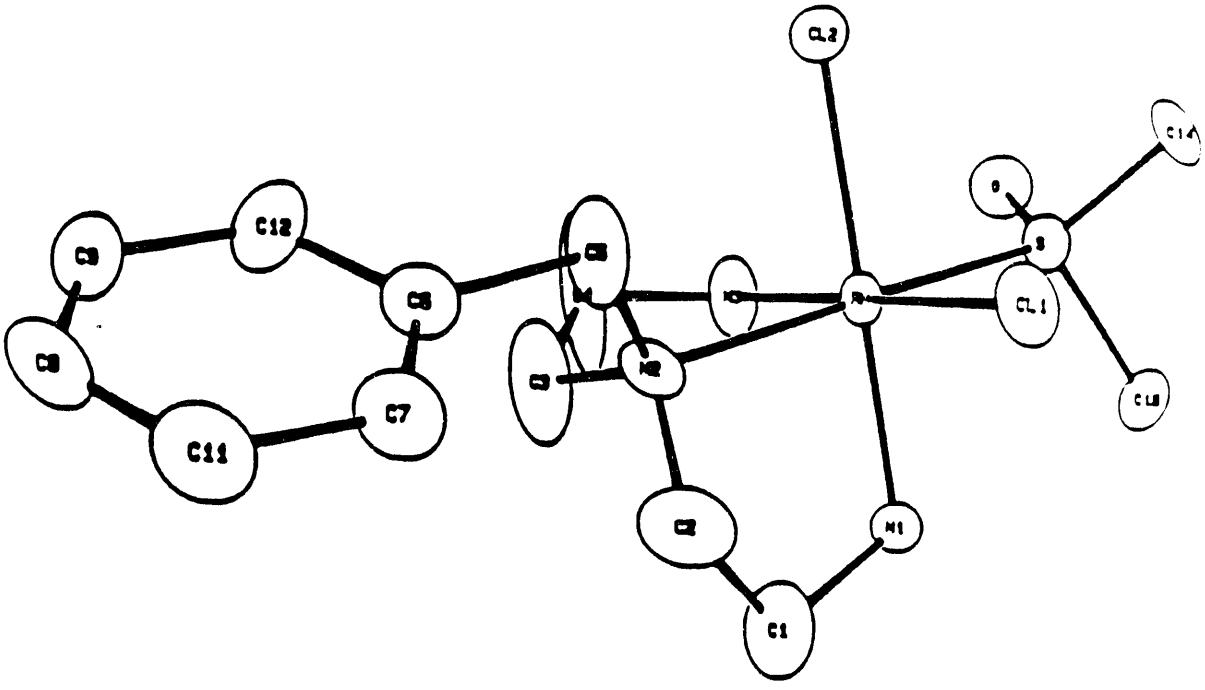


Fig. 2. Structures of rhodium complexes of the benzyl analogues of ligands I and III.

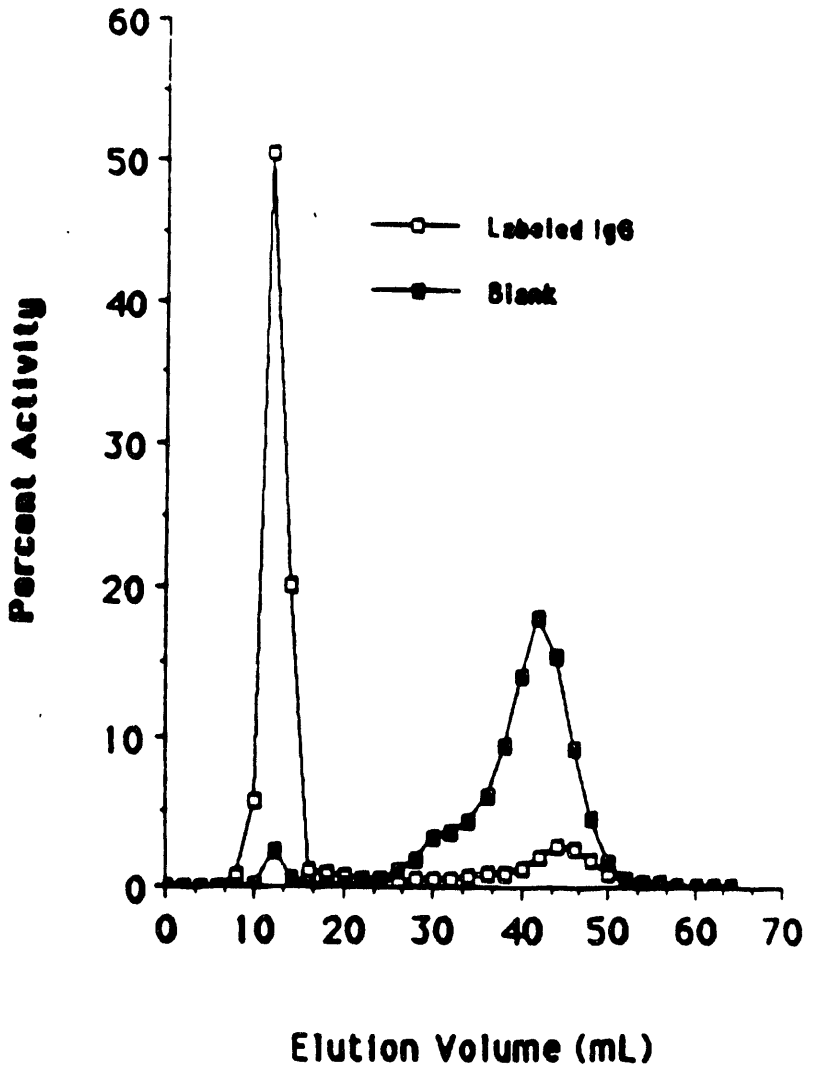


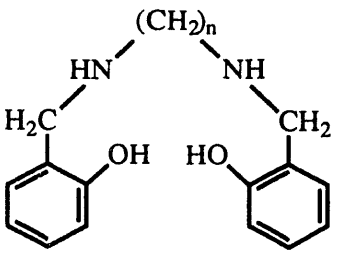
Fig. 3. Elution profile of a Rh-105-labeled antibody. The blank is for a run with no coupling agent (protein and unactivated complex). The first peak is labeled antibody, the second non-coupled Rh-105 complex.

Table 2. Coupling of Ligand V Complexes of Tc-99m, Cu-67 or -64, and Pd-109 to IgG

Radio-nuclide	Metal/ IgG	Incub. Time (hr)	Coupling Yield (%)	Atoms metal/ IgG
Tc-99m	0.1[a]	4	67	0.07[a]
	0.5		79	0.4
	1		77	0.8
	2		77	1.5
	5		71	3.5
Cu-67	1	1	69	0.7
or -64	5		30	1.5
	10		26	2.6
Pd-109	1	1	74	0.7
	2		75	1.5
	10		92	9.2

[a]Ratios for Tc-99m are based on ligand concentrations.

Since earlier studies (2) indicated that reduced amine oxime ligands gave higher yields and more stable and lipophilic complexes than the corresponding imine oxime ligands, we decided to prepare reduced forms (Figure 4) of ligand V and the analogs with variable chelate ring



VI

VIa n=2
VIb n=3
VIc n=4
VId $-CH_2C(CH_3)_2CH_2^-$

Fig. 4 Tetradentate amine phenol ligands.

sizes. Indeed the reduced forms produce more lipophilic Tc(V) complexes (Table 3) as indicated by chloroform extraction. This can probably be attributed to deprotonation of one amine nitrogen leading to neutral complexes.

Table 3. Comparison of Radiochemical Properties of Tc-99m Complexes of Ligand VI with Schiff Base Analogues

	Free TcO_4^- Percent		Percent $CHCl_3$ Extraction (% of Complex formed)	
Ligand	1 hr	24 hr	1 hr	24 hr
VIa	12	18	75	70
Schiff base	17	5	19	7
VIb	<1	<1	97	94
Schiff base	5	3	28	25
VId	<1	<1	99	99
Schiff base	17	10	43	39
VIc	4	7	94	94
Schiff base	25	13	45	48

Progress was made in obtaining substituted tetradentate amine oxime ligands such as that in the Rh(III) complex shown in Figure 5b. These neutral amine oxime complexes are known to be stable and inert, and functionalization of the benzyl group should allow protein conjugation.

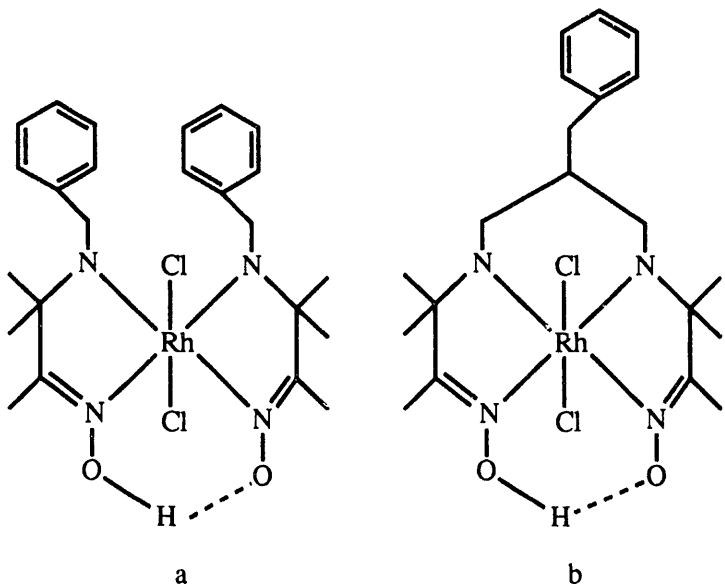


Fig. 5 Amine oxime complexes.

1989-91 Progress

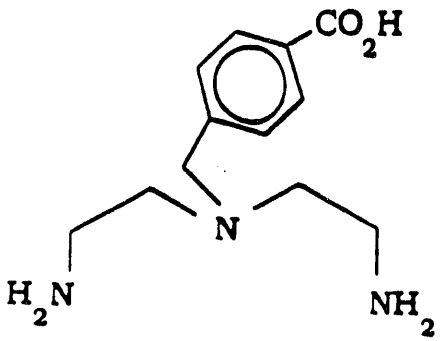
Progress during this period will be reported under the following headings:

1. Diethylenetriamine Based and Related Bifunctional Chelating Agents and their Complexation with Rh-105, Au-198, Pd-109, Cu-67, In-111, and Co-57.
2. Studies of Pd-109, Rh-105 and Tc-99m with Bifunctional Chelates Based on Phenylenediamine.
3. Establishment of an appropriate Protein Assay Method for Conjugated Proteins.
4. Studies of New Bifunctional Bi, Tri and Tetradentate Amine Oxime Ligands with Rh-105.
5. IgG and Antibody B72.3 Conjugation Studies by HPLC Techniques with Bifunctional Metal Chelates.
6. Progress on Ligand Systems for Au(III).

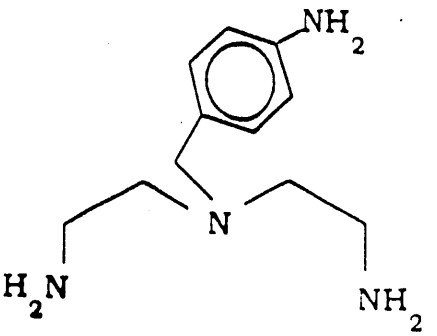
1. Diethylenetriamine Based and Related Bifunctional Chelating Agents and their Complexation with Rh-105, Au-198, Pd-109, Cu-67, In-111, and Co-57.

The ligands shown in Figures 6-9 were synthesized and characterized by proton NMR. Chelation of ligands 2 thru 6 with Rh-105, Au-198, Tc-99m, Pd-109, Cu-64 and 67, Co-57, and In-111 was studied in dilute solutions by radiochemical techniques. All isotopes were prepared at MURR by appropriate methods except Co-57 and In-111 which were obtained from Dupont Chemical Co. The aqueous complexation methods were standard and were described in one of our annual reports. Conjugation with the bifunctional chelate complexes was studied with human gamma globulin (Sigma 4386). Thiophosgene was used as the activating agent. Comparison was made with blanks (no thiophosgene). Protein bound complex was separated from free complex on G-75 Sephadex (gel permeation chromatography) for determination of conjugation yields. Table 4 summarizes the results for ligands **4b**, **5** and **6**. High complexation yields were obtained but extraction into $CHCl_3$ was poor (these complexes are probably charged) (extraction yields were better after activation with thiophosgene). Coupling yields were generally quite good. Coupling with ligands **5** and **6** was attributed to pyrrole moieties not directly bound to the metal. Thus Rh(III) complexes with free (unbound) pyrrole groups on the ligand backbone could potentially be used to provide conjugation to antibodies. The mechanism of this conjugation has not been established.

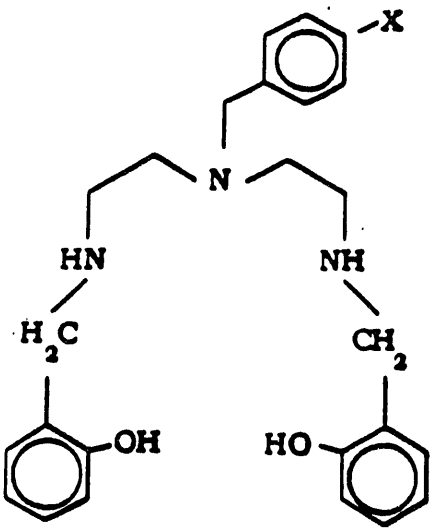
A comparison of complexation results of **2b**, **4b**, and **6** with the other metal ions is provided in Table 5. Generally the phenol ligand **2b** is a more effective chelator than the pyrrole ligands; however, none of the ligands gave satisfactory complexation with In-111. With Au-198 complexation with **2b** and **4b** was 60-80%, but protein labelling was just as effective with



1a



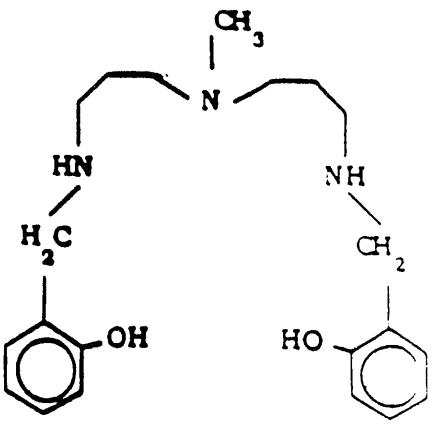
1b



2a X = NO_2

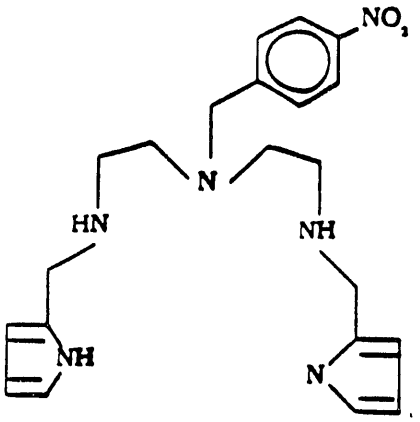
2b X = NH_2

2c X = H

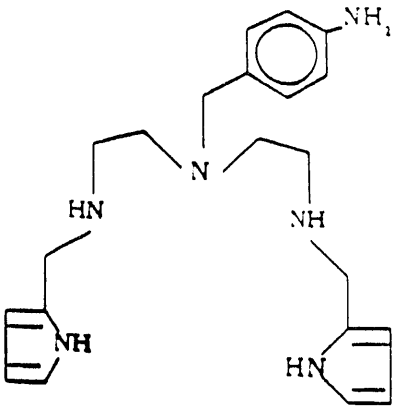


3

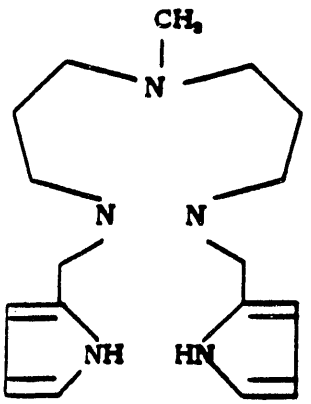
Fig. 6



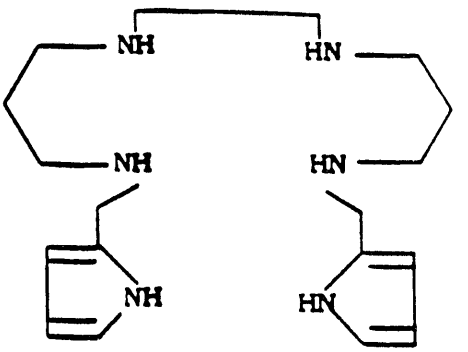
4a



4b



5



6

Fig. 7

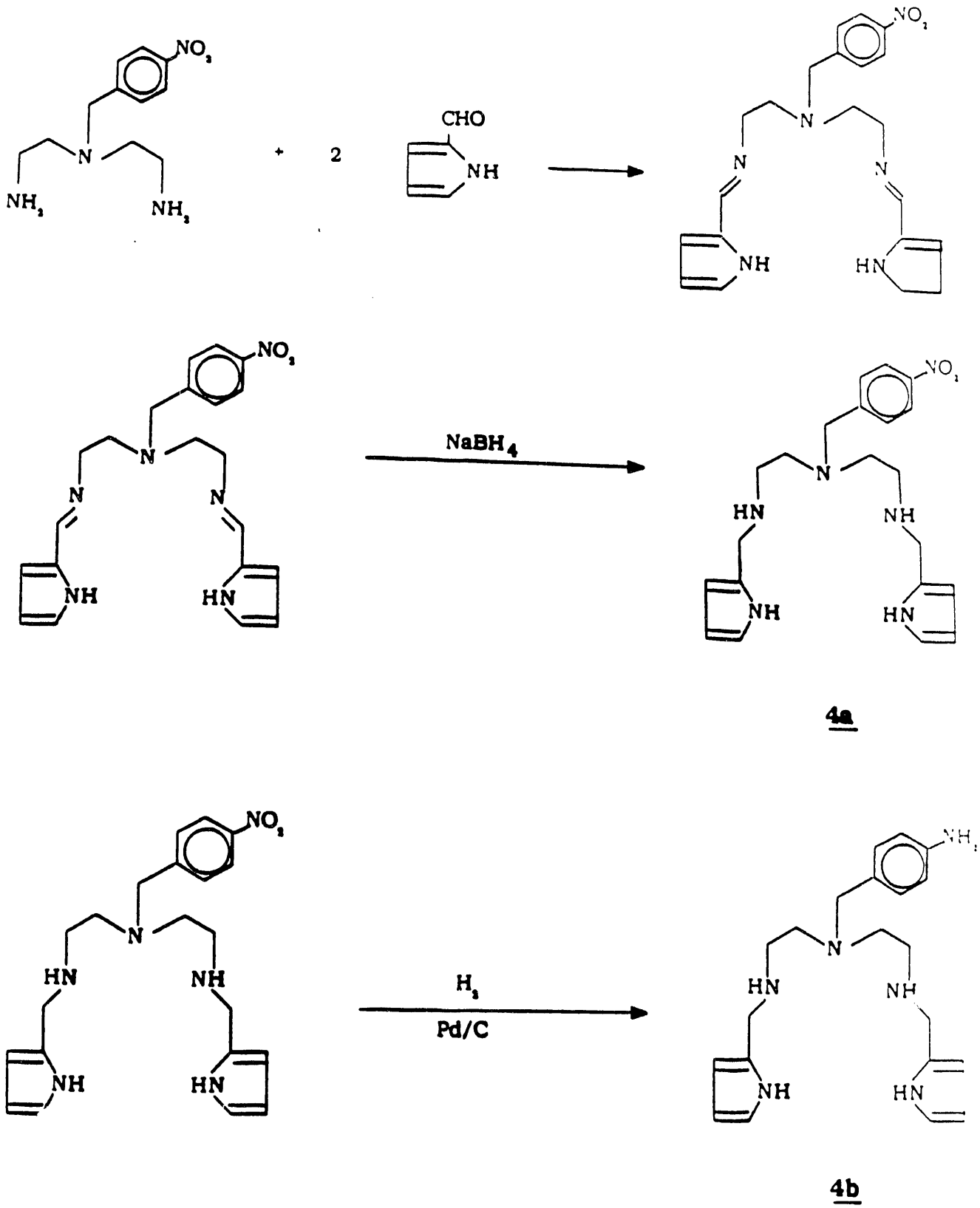


Fig. 8

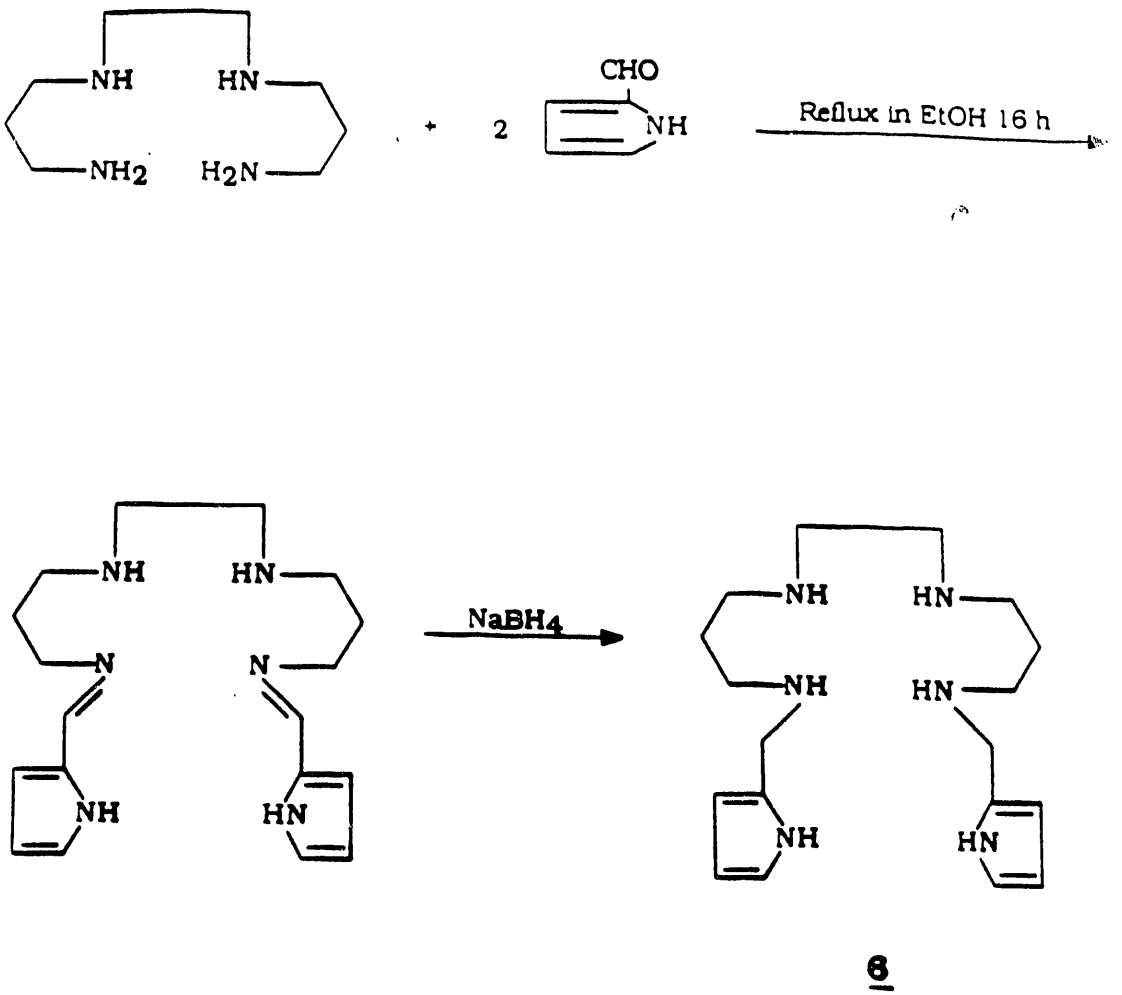

NH
HN
NH2
H2N
+
2
CHO
NH
Reflux in EtOH 16 h
NH
HN
N
N
NH
HN
NaBH4
NH
HN
NH
HN
NH
HN
6

Fig. 9

Table 4. Complexation and Coupling Yields with Rh-105

I.	Ligand	**4b**	**5**	**6**
II.	Complex yield, %	80	98	95
III.	Extraction in $CHCl_3$, %	28	21	18
IV.	Extraction of activated complex in $CHCl_3$, %	61	37	50
V.	Coupling yield, %	97	70	94
VI.	Overall coupling yield, %	60	26	47
VII.	Blank, %	16	7	4

[3] VI=IVxV

Table 5. Complexation and coupling yields (%)

	2b		**4b**		**6**	
Radionuclide	comp.	coup.	comp.	coup.	comp.	coup.
Tc-99m	+++	+++	++	++	++	++
Pd-109	+++	+++	a	a	a	a
Cu-64, 67	+++	+++	+++	++	+++	++
Co-57	+++	++	+++	++	+++	+
In-111	+	-	-	-	-	-

a Insoluble complexes were formed
+++ 80-100%
++ 50-80%
+ 20-50%
- <20%

unactivated as with activated complex probably indicating that transchelation occurs with direct labelling of the protein by the gold.

Preliminary experimentation with a linear tetraamine (1,4,8,11-tetraazanonane) (3) with Au(III) was carried out to see if direct labelling of the protein could be avoided. These experiments indicated formation of a cationic complex at ~80% yield. Only about 16% of the complex was found in the protein fraction after mixing with human gamma globulin and G-75 Sephadex separation of the free complex. Thus it appears that simple polydentate amine ligands might prove

useful for indirect (bifunctional) conjugation of Au(III) to proteins. A recent paper (4) on Au(III) complexes of cyclam(1,4,8,11-tetraazacyclotetradecane) and various derivatives suggests that ligands of that general nature might be used with appropriate functionalities.

2. Studies of Pd-109, Rh-105 and Tc-99m with Bifunctional Chelates Based on Phenylenediamine.

As another approach to bifunctional chelating ligands, the Schiff base ligand **7** was prepared by reaction of 3,4-diaminobenzoic acid with salicyclaldehyde, and the reduced analog **8** (Figure 10) was also prepared. Complexation with Rh-105 was better with ligand **8** (~83%) than with ligand **7** (~69%). The complexes were not efficiently extracted (30-40%) into $CHCl_3$ even at low pH (4). The complexes are probably negatively charged with loss of two protons from the ligand and coordination of two chloride ions. Good conjugation of the complex with **7** to human gamma globulin (nearly 100%) was obtained with isobutylchloroformate (ICBF). However, a blank experiment (no ICBF) showed 20% protein uptake.

In an experiment with Tc-99m using ligand **8**, the complexation yield was ~95% but attempted human gamma globulin conjugation was not very successful (only ~55% of the radioactivity in the protein fraction after G-75 Sephadex separation). Longer incubation with the protein, 20 hrs instead of 3, gave lower conjugation yield (40%) suggesting a decomposition problem.

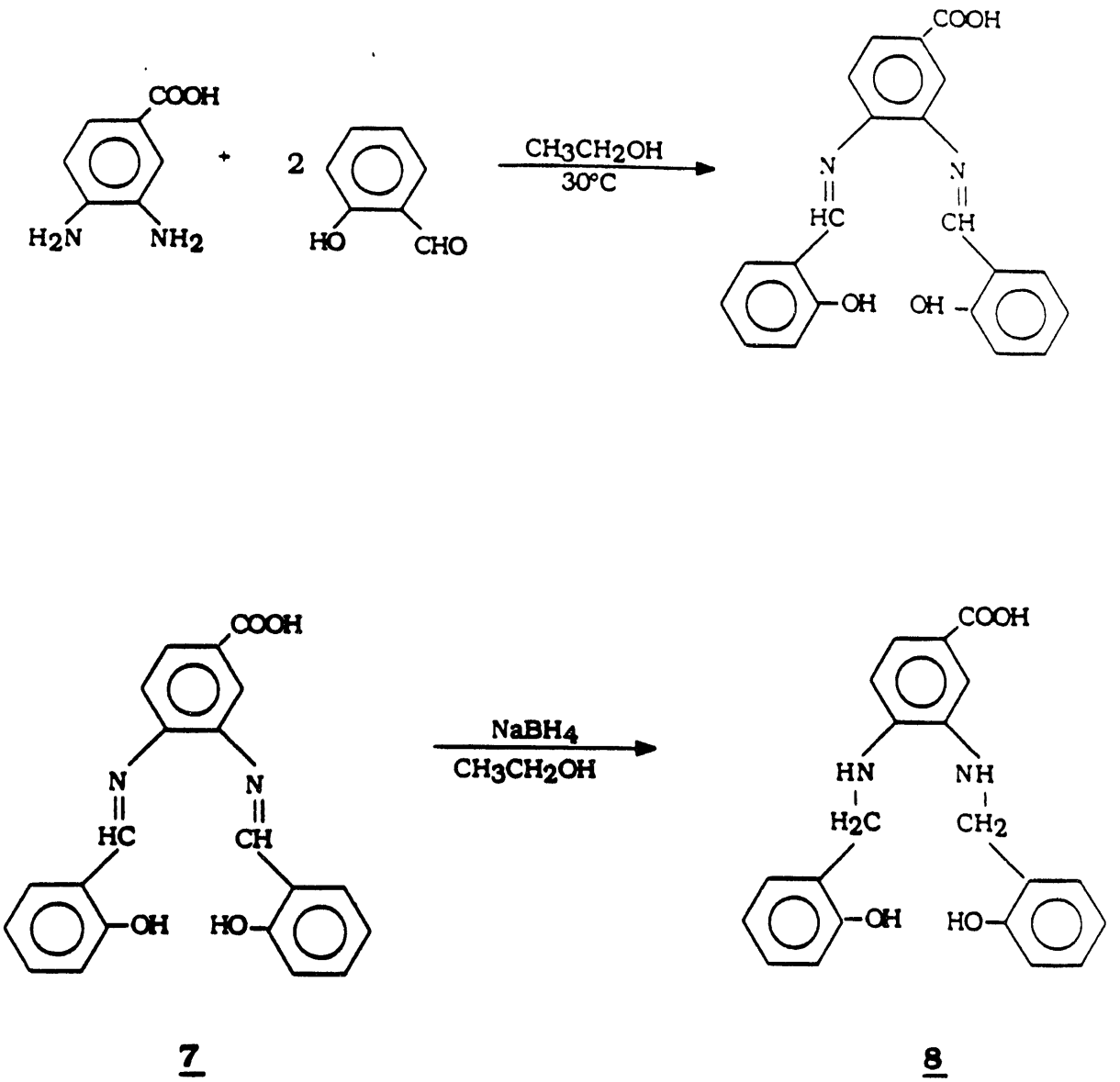
As with Tc-99m, complexation of Pd-109 was much more complete with the reduced ligand **8** (90-100%) than with **7** (50-60%) as indicated by extraction into chloroform or ethyl acetate (a neutral complex is expected here with Pd(II)). The complexation is carried out under basic conditions while the extraction is done after acidification (allows for protonation of the carboxyl groups). Figure 11 shows the yield as a function of time and ligand/Pd ratio.

3. Establishment of an Appropriate Protein Assay method for Conjugation to Proteins.

The purpose of the study was to establish a method to trace the amount of protein through conjugation studies. The Lowry procedure and the Bio-Rad Protein Assay procedure were examined for this purpose. The effects of buffers and ligands on the absorbance of the protein at various wavelengths was also studied.

Techniques

1. Lowry Procedure: An alkaline cupric tartrate reagent complexes with the peptide bonds and forms a purple-blue color when the phenol reagent is added (5). Absorbance is measured at a suitable wavelength between 500-800 nm. The protein concentration is then


COOH
H2N
NH2
+
2
HO
CHO
CH3CH2OH
30°C
COOH
N
HC
N
CH
OH
OH
COOH
N
HC
N
CH
OH
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NaBH4
CH3CH2OH
COOH
HN
H2C
NH
CH2
OH
HO
7
8

Fig. 10

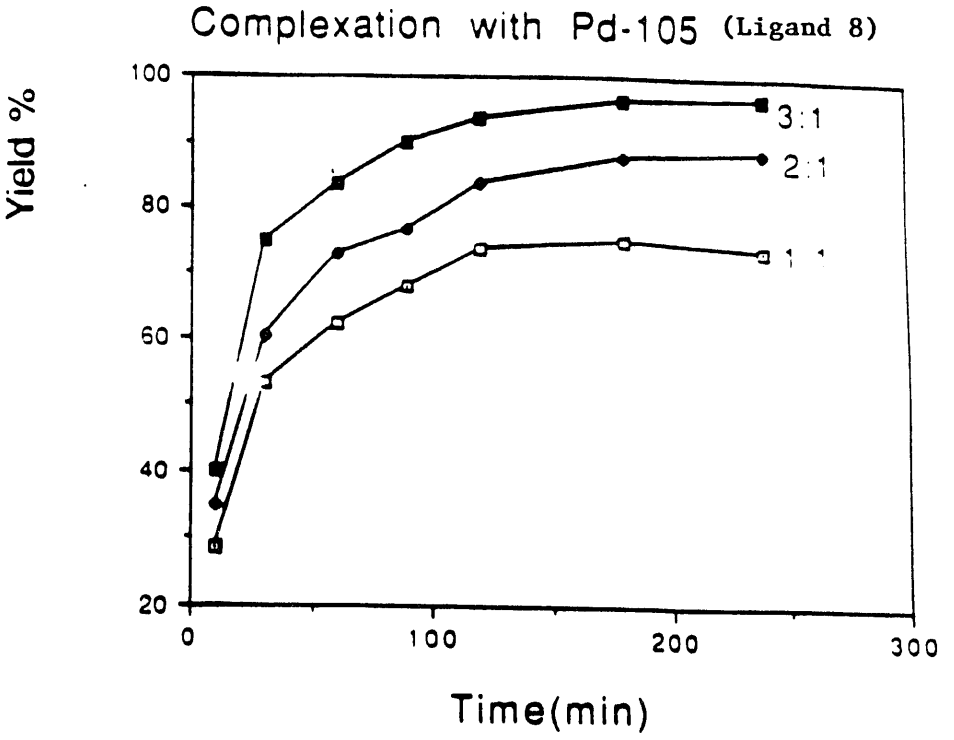

Complexation with Pd-105 (Ligand 8)
Yield %
100
80
60
40
20
0
100
200
300
Time(min)
3:1
2:1
1:1

Fig. 11

determined from a calibration curve.

2. Bio-Rad Protein Assay: This method is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 to 595 nm when binding to a protein occurs (6).

Experiments

A number of experiments with the Lowry method were done at 750 nm: 1) effect of tris buffer concentration on HSA absorbance; 2) effect of borate buffer concentration on HSA absorbance; 3) effect of saline on BSA absorbance; 4) effects of concentrations of various ligands on HSA absorbance; and 5) assay of HSA, IgG and BSA after elution through short columns (prepacked G-25 Sephadex).

For the Bio-Rad method studies included: 1) column saturation effects; 2) effects of "free" palladium (cold=nonradioactive) on protein absorbance; 3) effect of ligands on IgG absorbance; and 4) effect of Tc(V) ligand complex on IgG absorbance.

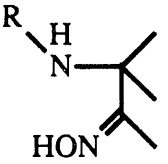
Finally the effect of conjugation on the protein assay was studied using both short (10 cm) Sephadex G-75 columns and 30 cm columns.

Conclusions

1. Tris buffer interferes with the measured absorbance of the protein much more than the borate buffer especially at higher buffer concentration. Saline, however, has no effect on the absorbance of the protein
2. Ligands with phenol groups interfere significantly with the protein absorbance.
3. In all cases, protein elution through the columns resulted in less than 100% recovery of the protein. A certain amount seemed to adsorb on the column.
4. The absorbance of palladium conjugated to the protein seemed to have a negligible effect on the absorbance of the protein.
5. About 90% of the recovered protein is eluted in the second void volume of the column. The radiolabel was eluted with the protein; the unconjugated complex was eluted later.
6. Bio-Rad Protein Assay is much easier to use than Lowry Assay. The former requires only one reagent and only 5 minutes to perform. It is also more convenient to use with micro samples since it requires only 0.2 mL of the reagent, keeping the entire volume small.
7. Most importantly the Bio-Rad method was found to be free from the interferences observed with the Lowry method.

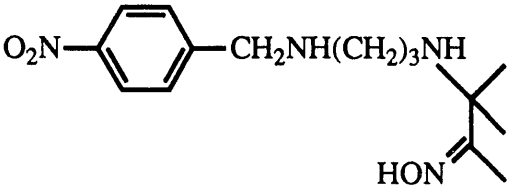
4. Studies of New Bifunctional Bi-, Tri-, and Tetradentate Amine Oxime Ligands with Rh(III) (Rh-105).

A large number of new bidentate amine oxime ligands of the type

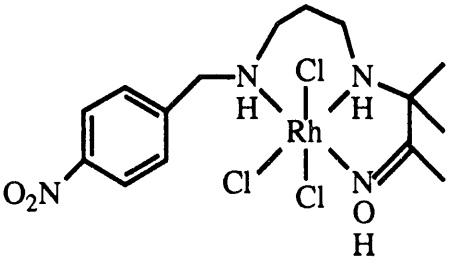


have been synthesized and characterized (7). The structures of most of their Rh(III) complexes have been determined and most have the general structure illustrated by R=benzyl (Figure 2) (8). The corresponding complex with R=4-aminobenzyl was used in studies of protein conjugation with the complex of Rh-105 (8). The complexation with Rh-105 at pH=9 in HCO_3^- buffer was greater than 95%. No loss of activity as inorganic rhodium during complexation was observed. The complex formed is soluble in water up to at least 10^{-3} M. Near quantitative conversion of the amine to the isothiocyanate with thiophosgene was observed and this complex can be nearly quantitatively extracted into chloroform, leaving behind essentially all other rhodium species. By varying the concentration ratio of the complex to γ-globulin (0.46-2.7) we were able to obtain conjugation of 0.35-2 rhodium atoms per molecule of γ-globulin (an average of about 85% conjugation yield was observed). A blank with unactivated complex gave about 10% conjugation. Thus this ligand provides a promising approach to conjugation. Possible aggregation of the proteins due to two binding sites per complex is being investigated.

For the tridentate case we have so far achieved synthesis of the (4-nitrobenzyl) amine oxime ligand:



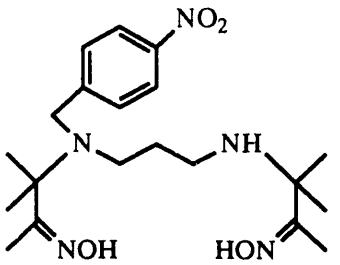
This ligand produces a rhodium(III) complex whose structure from x-ray diffraction analysis is



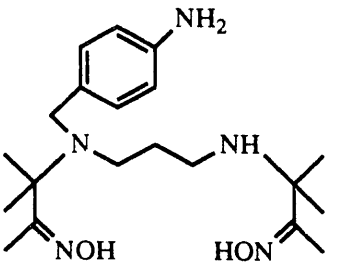
We plan to reduce the nitro group to an amine in order to study its conjugation to proteins.

Our efforts to prepare tetradentate bifunctional ligands which would bind to a Rh(III) metal center and contain a functional group able to couple to a monoclonal antibody have led to the development of the following two ligand systems:

1)

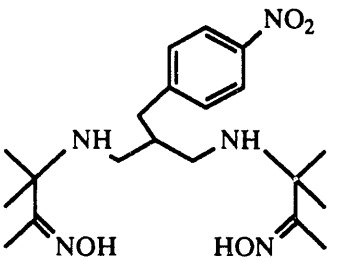


I

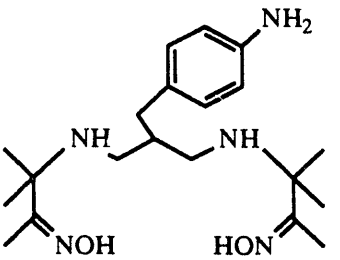


II

2)



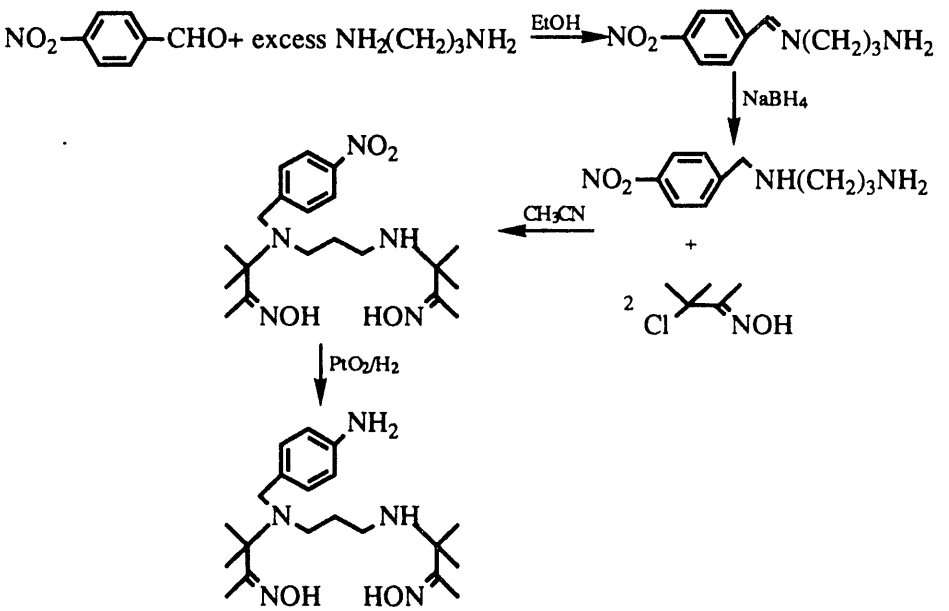
III



IV

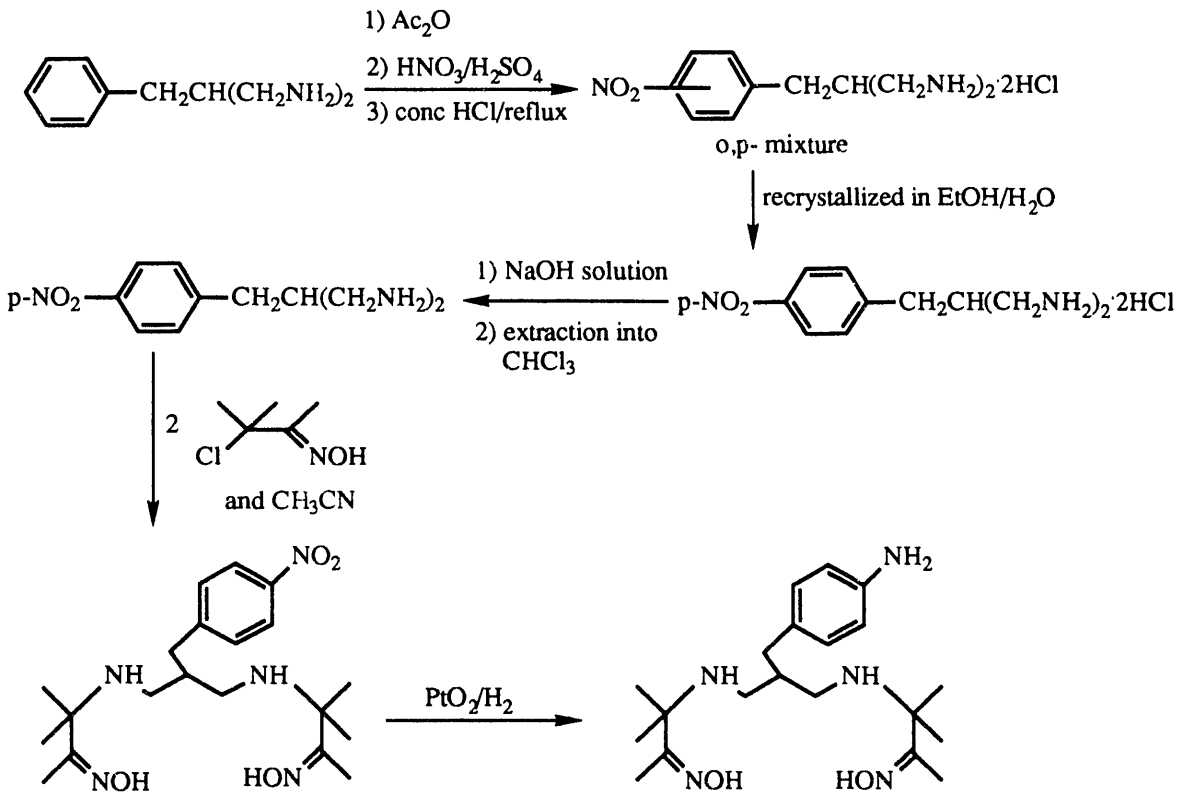
The ligands with the aromatic NO_2 functionality (**I** and **III**) were prepared in order to have a functional group which could easily be reduced to the NH_2 moiety (**II** and **IV**). This moiety could then be activated with thiophosgene in order to link the complexes of these ligands to monoclonal antibodies.

The synthetic scheme for the first set of ligands is shown below:



The last step in this synthetic route has been attempted numerous times varying the H_2 pressure and time of reaction along with the hydrogen uptake. The NMR data on the resulting unpurified product (purification at this point is difficult if not impossible) gives every indication that the NO_2 moiety has been reduced; however, upon complexing this product with Cu^{2+} or Ni^{2+} and conducting an X-ray diffraction study on a crystal of the complex, the aromatic portion of the molecule was absent. Apparently, the carbon-nitrogen bond which connects the aromatic ring to the rest of the ligand must cleave under these reaction conditions, and thus other catalysts and conditions are being explored.

For the second ligand system where the benzyl moiety is attached to an aliphatic carbon rather than the amine nitrogen the reduction of the NO_2 group proceeded to the ligand with the desired NH_2 group. The synthesis of these ligands were done as follows:



The above ligands were characterized by NMR. Radiochemical experiments (Rh-105) were performed using the ligand with the aminobenzyl group on the central carbon of the propylene backbone of the amine oxime ligand. The details of these experiments are discussed below.

In the radiochemical experiments conducted with the aminobenzyl group on the propylene backbone, the main objective has been to optimize the complexation of this ligand with the Rh-105 isotope while maintaining the high extraction of the resulting complex into $CHCl_3$. Because the complex is neutral it should be extracted into the organic $CHCl_3$ layer from the aqueous reaction mixture; the complex is then activated with thiophosgene. After activation the $CHCl_3$ is removed

and the activated complex is reacted with the antibody.

Some of the parameters that needed to be varied in order to improve the complex yield were: pH of the reaction mixture, ligand concentration and the amount of ethanol present during complexation. The complex yield was greatest under higher pH conditions, with higher ligand concentrations and in about a 1:1 ethanol-water mixture. Too much ligand present during the complexation procedure was also a problem, because the uncomplexed ligand is also extracted into the $CHCl_3$ layer and activated along with the complex. If a large excess of the ligand is present, then the activated free ligand competes with the complex during the coupling step; this competition lowers the coupling efficiency of the complex and potentially the immunoreactivity of the antibody.

A solution of this ligand was made by dissolving .0193 g of the ligand in 10 mL of absolute ethanol. The resulting ligand solution (5.11×10^{-3} M) was used for all of the complexations described below.

A variety of reaction mixtures were formulated to optimize the percentage yield of the Rh-105 complex formed with ligand IV and the percentage of the complex extracted into chloroform.

All of the formulations contained the ligand (in EtOH), Rh-105 (an acidic aqueous solution from the research reactor), absolute ethanol and saline solution. The pH of the resultant solutions was adjusted using 0.1 M NaOH and 0.1 M HCl. The pH range giving the best overall complexation and $CHCl_3$ extraction results was between 4-6. If the pH exceeded 7, the complexation was generally better, but the extraction poorer.

Table 6 gives formulations used to determine the best conditions for complex formation and $CHCl_3$ extraction.

Table 6

Formulation	Rh-105	Ethanol	[a]NaOH	[b]Ligand
1	0.2 mL	0.8 mL	1.0 mL	0.5 mL
2	0.5 mL	0.4 mL	1.5 mL	0.1 mL
3	1.0 mL	1.0 mL	2.4 mL	0.1 mL
4	1.0 mL	2.0 mL	2.2 mL	0.1 mL
5	0.5 mL	1.0 mL	1.0 mL	1.0 mL

[a]The amount of 0.1 M NaOH needed to adjust the pH to approximately 5-6. One exception was in formulation 4: the pH was approximately 9. Formulation 1 contained about 0.5 mL of a 0.9% saline solution as part of the 0.1 M NaOH volume.

[b]The ligand concentration was approximately 5×10^{-3} M.

The components of the reaction mixtures in the above table were combined in the following order:

1. The Rh-105 solution was added to a 10 mL round-bottom flask.
2. The NaOH solution was then added to adjust the pH.
3. The remaining reagents were added.

The reagents were mixed and the reaction solutions were refluxed for varying amounts of time. Aliquots were taken at various times to determine the extent of reaction. Table 7 gives the reaction times, yields and the % $CHCl_3$ extraction of these complexes for the various formulations:

Table 7

Formulation	Reaction Time	Complex Yield	Extraction %
1	80 minutes	64.5%	56.3%
1	110 minutes	69.7%	63.8%
2	65 minutes	70.9%	64.8%
3	80 minutes	--------	36.6%
4	120 minutes	85.7%	28.1%
5	90 minutes	79.1%	58.5%

The complexes were activated with a $SCCl_2$ solution (10 μL of the concentrated $SCCl_2$ from Aldrich was added to 1 mL of $CHCl_3$). Varying amounts of this activating solution were added to the aqueous reaction solution. The mixtures were vortexed vigorously for approximately 2 min and then extracted with $CHCl_3$. Aliquots from each layer were counted in order to determine the amount of the activated complex extracted into the organic phase. Table 8 shows the results of the $CHCl_3$ extractions of the activated complexes.

Table 8

Formulation	$SCCl_2$ solution	Complex[c]	Extraction (%)
1	50 μL	1.0 mL	80.9%
2	50 μL	1.2 mL	81.9%
3	50 μL	1.2 mL	72.0%
4	50 μL	1.0 mL	79.1%
5	100 μL	1.2 mL	88.4%

[c]The complex volumes that contained 1.2 mL of solution consisted of the reaction solution and 0.2 mL of 0.1 M NaOH. All of the aqueous reaction solutions were extracted with an equivalent amount of chloroform.

Since the activated complex was easily extracted into chloroform in most cases, it was available for conjugation without further purification. Formulations 2, 3, 4 and 5 were selected to examine the degree of conjugation with IgG and B72.3 antibody. The procedure for conjugation was similar for all of the reaction solutions.

An aliquot of the chloroform extract was placed into a small test tube. The organic solvent was removed by evaporation under a stream of air. The residue was reconstituted in dimethylformamide (DMF) and an aliquot of this solution was placed in a test tube. The desired antibody solution was then added while the test tube was mildly agitated. These conjugation solutions were then allowed to react for various times.

The conjugation yields were determined by loading an aliquot of the conjugation solutions onto a size exclusion column containing G-75 Sephadex gel permeation packing material in saline. A 0.9% (w/w) aqueous saline solution was used as the mobile phase to elute the activity from the column; aliquots were collected every 1.8 minutes into small test tubes. These fractions were counted with a NaI detector. The activity associated with the antibody was eluted from the column first. The remaining activity was considered unconjugated complex.

Table 9 shows the conditions of the conjugation experiments conducted on formulations 2, 3, 4 and 5 of Table 6:

Table 9

Formul. Number	Aqueous Volume[a]	Organic Volume[b]	DMF Used	Complex Volume	Antibody/ Volume[c]
2	1.0/2.0	1.0-1.1	20 μL	10 μL	IgG/0.2
2	" "	" "	" "	10 μL	B72.3/0.2
3	1.0/3.4	1.0-1.1	15 μL	10 μL	IgG/0.2
3	" "	" "	" "	5 μL	B72.3/0.2
4	1.0/3.2	1.0-1.1	10 μL	10 μL	IgG/0.5
5	1.0/1.0	1.0-1.1	10 μL	10 μL	IgG/0.5

[a]These volumes are in mL and reflect the amount of the reaction solution used versus the total volume that was available.

[b]These volumes are in mL and reflect the amount of the organic layer recovered when 1 mL of the reaction solution and 0.2 mL of 0.1 M NaOH was extracted with 1.2 mL of chloroform.

[c]The antibody solution volume reacted with the DMF solution of the complex given in mL. The concentration of both the IgG and B72.3 antibody solutions was 0.5×10^{-5} M.

After all of these solutions were prepared, the conjugation yields were determined chromatographically. Table 10 includes the formulation number used with its respective antibody,

the ligand:antibody ratio, the reaction time before introduction onto the column and the resulting conjugation yields:

Table 10

Formulation/ Antibody[a]	Ligand: Antibody[b]	Reaction Time	Conjugation Yield
2/IgG	12.5:1	74 hr	81.8%
2/B72.3	12.5:1	44 hr	61.0%
2/B72.3	12.5:1	65 hr	64.8%
3/IgG	10:1	134 hr	90.2%
3/B72.3	5:1	<24 hr	74.0%
4/IgG	6.2/1	23 hr	88.0%
5/IgG	20/1	4 hr	64.0%
5/IgG	20/1	24 hr	83.6%

[a]The amount of radioactivity loaded onto the column was at least 47,000 counts per 10 seconds. The volume of complex was between 20 μL and 100 μL.

[b]The ligand concentration was determined using the amount of ligand (0.5×10^{-3} mmol) added to the total volume of the solution. The antibody concentration was determined using the volume of the antibody solution reported and the molarity of the antibody solution (5.0×10^{-5} M).

Two additional reaction solutions were used to study the conjugation of this complex with protein as a function of time. One reaction solution contained nonradioactive Rh(III) (5.0×10^{-3} M) in the formulation. The addition of this cold rhodium allowed the establishment of a ligand:metal ratio in to demonstrate the ligand's ability to complex at a low ligand:metal ratio. Table 11 shows the reactants for these formulations:

Table 11

Formulation	Rh-105	Ethanol	NaOH[a]	Ligand
6	0.5 mL	0.8 mL	1.1 mL	0.1 mL
7[b]	0.5 mL	0.65 mL	1.1 mL	0.25 mL

[a]The volume for formulation 6 was a combination of 0.5 M NaOH and 1 M HCl required to adjust the pH to approximately 5-6 and 0.9% (w/w) saline. The volume for formulation 7 was a combination of 0.5 M NaOH, 0.1 M NaOH and 1 M HCl required to adjust the pH to approximately 5-6 and 0.9% (w/w) saline.

[b]Formulation 7 had 0.1 mL $RhCl_3 \cdot 3H_2O$ (5×10^{-3} M) added to the reaction solution in order to have a ligand:metal ratio of 2.5:1. The ligand was added to the reaction solution after the Rh

mixture had refluxed for 15 minutes.

After these reaction mixtures were refluxed the complex yield and the % extraction into $CHCl_3$ were determined as described above. Table 12 gives the results:

Table 12

Formulation	Reaction Time	Yield	% Extraction into $CHCl_3$
6	110 min	73.9%	55.7%
7	110 min	65.9%	66.9%

The activated complexes of formulations 6 and 7 were prepared using the procedure described above for the other formulations. Table 13 contains the % $CHCl_3$ extraction of these activated complexes:

Table 13

Formulation	$SCCl_2$ Solution	Complex	% Extraction into $CHCl_3$
6	50 μL	1 mL	69.0%
7	100 μL	1 mL	88.9%

Once effective extraction of these complexes was established, the activated complex was recovered as described previously and reacted with both IgG and B72.3. Table 14 gives the conditions used for these reactions:

Table 14

Formulation	Aqueous Volume[a]	Organic Volume[b]	DMF Used	Complex Volume[c]	Antibody/ Volume
6	1.0/2.0	0.5 mL	25 μL	5 μL	IgG/0.5
6	" "	" "	" "	5 μL	B72.3/0.5
7	1.0/2.0	0.5 mL	25 μL	5 μL	IgG/0.5
7	" "	" "	" "	5 μL	B72.3/0.5

[a]The volume of each reaction mixture was adjusted to 2.0 mL with 0.1 M NaOH and then 1.0 mL of the resulting solution was extracted with 1 mL of chloroform.

[b]0.5 mL of the organic phase was placed in a small test tube, the solvent was removed and the residue was reconstituted in 25 μL of DMF.

[c]5 μL of the DMF activated complex solution was reacted with the proteins.

These conjugation solutions were allowed to react for varying amounts of time before the conjugation yields were determined by chromatographing an aliquot of the reaction mixture on a size exclusion column. All of the above conjugation reactions were followed over time to determine the optimum reaction time. Table 15 contains the reaction time versus complex yield data:

Table 15

Antibody/ Formula[a]	Total Ligand/ Antibody[b]	Reaction Time	Conjugation Yield
IgG/6	1:1	2.5 hr	46.1%
IgG/6	1:1	7.5 hr	89.3%
IgG/6	1:1	17.0 hr	92.4%
IgG/6	1:1	40.5 hr	94.1%
B72.3/6	1:1	1.5 hr	20.7%
B72.3/6	1:1	6.5 hr	37.8%
B72.3/6	1:1	16.0 hr	50.8%
B72.3/6	1:1	37.5 hr	67.8%
IgG/7	2.5:1	5.0 hr	83.9%
IgG/7	2.5:1	9.5 hr	88.7%
IgG/7	2.5:1	19.5 hr	90.7%
IgG/7	2.5:1	42.5 hr	92.0%
B72.3/7	2.5:1	4.0 hr	28.5%
B72.3/7	2.5:1	8.5 hr	39.1%
B72.3/7	2.5:1	18.0 hr	50.8%
B72.3/7	2.5:1	41.5 hr	65.6%

[a]50 μL of each of these solutions was loaded onto the size exclusion column that contained Sephadex G-75 equilibrated in saline.

[b]The ligand concentration was determined from the original $5x10^{-4}$ mmol of ligand added and using the ratio of the volume used:total volume along with fact that only half of the organic layer was used. Therefore, only $1.25x10^{-4}$ mmol of ligand remained in the DMF solution. A 1:5 dilution was done since only 5 μL of the 25 μL available was used. Consequently, $2.5x10^{-5}$ mmol was reacted with $2.5x10^{-5}$ mmol of the protein (0.5 mL of $5x10^{-5}$ M) giving a 1:1 ratio. Formulation 7 had 2.5 times as much ligand which resulted in the 2.5:1 ratio. This increased ratio was used because of the carrier added nonradioactive Rh (thus a 2.5:1 ratio of ligand to Rh).

The conjugation yield versus reaction time data is shown graphically in Figure 13.

One final radiochemical experiment was done to determine if the conjugation of this particular complex to the B72.3 antibody reduced the immunoreactivity of the antibody. This experiment involved the formation of the activated complex and conjugation of this complex to the B72.3 protein. The first part of this procedure involved the same steps previously reported.

A complex solution was prepared containing the following ingredients:

0.5 mL Rh-105
1.4 mL 0.05 M NaOH
0.5 mL ethanol
0.1 mL ligand $5x10^{-3}$ M

The pH of this reaction solution was approximately 5-6. The reaction mixture was refluxed for 1 hr; the reflux condenser was removed during the last 15 minutes so that the ethanol would evaporate. The MgO experiment gave a complexation yield of 76.2%. 38.5% of this complex could be extracted into the chloroform layer before activation. However, when 1 mL of the complex solution was made basic with 0.2 mL of 0.5 M NaOH, activated with 100 µL of $SCCl_2$ solution and then extracted with 1.1 mL of $CHCl_3$, 73.0% of the activated complex was recovered in the organic phase. The organic phase was transferred into a small test tube, the solvent was removed with a gentle stream of air, and the residue was reconstituted in 40 µL of DMF. A 5 µL aliquot of this DMF solution was reacted with 0.5 mL of $4.2x10^{-5}$ M B72.3 antibody. The total ligand:antibody ratio was 2:1. A 50 µL aliquot was loaded onto a size exclusion column after 4 hr of incubation. The conjugation yield was 42.5%. After a reaction time of 7 hr 25 minutes, the conjugation yield was 54.5%, and after 17.5 hr the conjugation yield reached 61.9%.

Immunoreactivity was studied on the above reaction mixture using the antigen of B72.3 on an appropriate short column. The conjugated complex was <u>not</u> separated from the rest of Rh(III) complex before addition. If the immunoreactivity of the B72.3 antibody was not affected by conjugation then the % activity corresponding to conjugation yield above should remain on the column, i.e., associated with the antigen.

50 µL aliquots of the conjugation mixture were loaded on appropriate antigen columns after 6 hr 10 min and after 18 hr of conjugation reaction time. In each case 2 mL of a 0.9% saline solution was used to wash off any free activity. In the first case 48.6% of the activity remained on the column while after 18 hrs 62.8% was found on the columns. These are essentially the % conjugation expected for these reaction times based on the conjugation data at 4 hr, 7 hr 25 min, and 17.5 hr of 42.5%, 54.5%, and 61.9%. Thus essentially 100% of the conjugated complex is associated with immunoreactive B72.3.

The radiochemical studies with this ligand system can be summarized as follows:

1. Complex yields of 65-86% were obtained within 2 hrs of reaction at pH=4-6.
2. At that pH extraction of the unactivated complex in $CHCl_3$ was poor (28-64%).

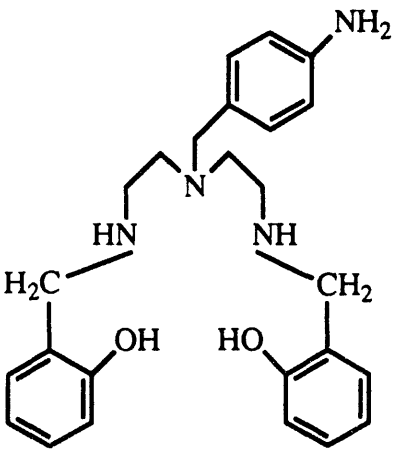
However, if the aqueous mixture was first activated with thiophosgene in a biphasic mixture with $CHCl_3$, the % extraction increased to 72-88%.

3. Conjugation studies of these activated complexes from the $CHCl_3$ extracts with IgG and antibody B72.3 were done as a function of time as well as ligand to antibody ratio. Excellent yields could be obtained in a few hours (5-8 hrs) even at low ligand/antibody ratios with IgG (Figure 13). Complete reaction with B72.3 appears to require a much longer time (only about 66% conjugation after 40 hrs) (Table 15).
4. Immunoreactivity studies on antigen columns indicated essentially 100% retention of activity for the conjugated B72.3.
5. The results of some preliminary HPLC studies on the protein conjugate of the Tc-99m complex of this ligand are included in the next section and are also very promising.

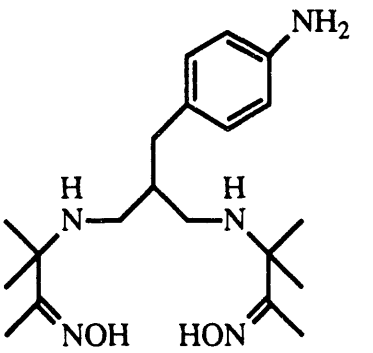
Thus it appears that this system provides a viable approach to labeling antibodies for possible *in vivo* studies. Future work with this complex is proposed for the next grant period.

5. IgG and Antibody B72.3 Conjugate Studies by HPLC Techniques with Bifunctional Chelates.

The ligands below, which were reported in earlier sections, were used for this study of HPLC characterization of conjugation.



Ligand 1



Ligand 2

I. Preparation of Labeled Proteins:

A. Technetium

i. Complexation

Complex conjugation with protein vs. time study with RhNCSPNAO

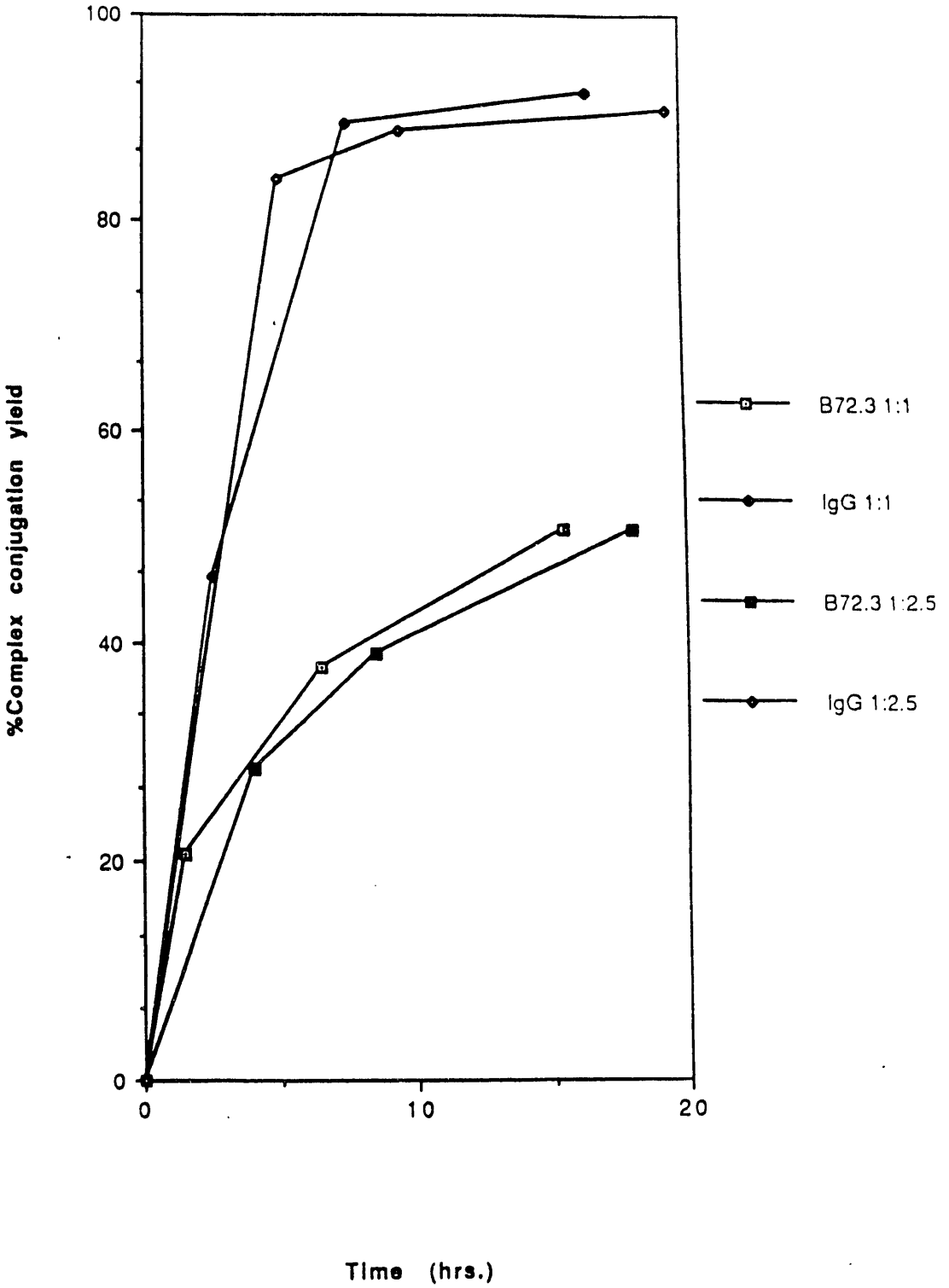


Fig. 13

The technetium complexes were prepared using known concentrations of Tc-99 as NH_4TcO_4 spiked with 99m-TcO_4^-. The ligands were dissolved in aqueous solutions of ethanol. A saturated solution of stannous tartrate, which had been prepared by stirring ~50 mg of the solid in 10 mL of saline solution and purging with nitrogen for ~15 min, was used as the reducing agent. The typical constituents and volume for the technetium complexation are as follows:

Sample	Volume (mL)
0.5 M $NaHCO_3$	1
normal saline	1.7
$5x10^{-4}$ M ligand*	1
10^{-4}-10^{-7} M NH_4TcO_4*	1
$^{99m}TcO_4^-$	0.1
Sn^{+2} (Sat.)	0.2

*Concentrations were adjusted to vary ligand and $^{99}TcO_4^-$ ratios

ii. Conjugation

A 1 mL solution of the complex was vortexed with 1 mL of $CHCl_3$ for 1 min. The chloroform layer which contained the neutral Tc complex was separated, placed in another tube and mixed with 0.01 mL of thiophosgene solution (0.13 M in $CHCl_3$) on a vortex mixer for 1 min. for activation. The solution was evaporated in a warm water bath under air. The residue was dissolved in 0.1 mL DMF and added to 2 mL of $5x10^{-5}$ M γ-globulin solution (borate buffer at pH 8.5) to complete the conjugation.

B. Rhodium (Rh-105) with Ligand 1

i. Complexation Study

a. Complexation

1 mL of saline, 0.5 mL of 0.5 M bicarbonate buffer (pH 9), 0.1 mL of Rh-105 solution and 0.1 mL of $1x10^{-3}$ M $RhCl_3 \cdot 3H_2O$ (nonradioactive) were refluxed for 15 min In a 10 mL round bottom flask fitted with a condenser. 1 mL of $1.25x10^{-3}$ M ligand was added, and refluxing was continued for 1.5 hr.

b. Characterization of the Complex

The complex yield was determined by the magnesium oxide adsorption technique. This method relies on the fact that free rhodium ions complexed with Cl^- and/or H_2O and carbonate complexes adsorb on the MgO while the organic complexes do not (9). The complex solution, (50 µL), was diluted to 0.4 mL in saline and mixed with ~50 mg of magnesium oxide powder. This mixture was vortexed for 1 min and centrifuged. 0.2 mL of the supernatant was transferred to

another test tube, and the two fractions were counted separately. The percent complex yield was obtained by dividing the activity in the total volume of supernatant by the total activity times 100. The assumption is that all Rh(III) in the supernatant is complexed with the added ligand while the uncomplexed Rh(III) remained with the MgO.

Extraction of the complex into $CHCl_3$ was determined by mixing 1 mL of the complex with 1 mL of chloroform on a vortex mixer for ~1 min. 0.2 mL of each layer was transferred to separate test tubes and the radioactivity of each was counted.

Blanks for the Rh-105 complex were prepared in bicarbonate buffer at pH 9 and in saline solution. The first was prepared by mixing 2.9 mL saline solution, 0.5 mL of 0.5 M bicarbonate buffer, 0.1 mL of 1×10^{-3} M $RhCl_3 \cdot 3H_2O$ solution, 0.1 mL Rh-105 in a 10 mL round bottom flask and refluxing for 15 min. 50 mL of this solution was transferred to a test tube and characterized by the MgO method. The second blank was prepared by mixing 0.395 mL saline solution, 5 mL Rh-105 on a vortex mixer for ~1 min, then 50 μL of it was analyzed with the MgO method.

ii. Conjugation

A solution containing 1 mL of the Rh complex, 0.9 mL of $CHCl_3$, and 0.1 mL of thiophosgene solution (0.13 M in $CHCl_3$) was vortexed for ~1 min. The organic layer containing the activated complex was separated into another tube and dried under air in a water bath. The residue was dissolved in 20 μL of DMF and mixed with the protein solution.

iii. Characterization of the Coupled Protein

At various times of incubation, 0.1 mL of the reaction solution was eluted through a Sephadex G-75 gel filtration column, and the yield of the coupled protein was estimated by comparing the radioactivity eluted with the protein to the total radioactivity applied to the column. Blanks for coupling the complex to the protein were prepared in the same way as for conjugation, but without the addition of the $CSCl_2$.

II. HPLC

20 μL aliquots of the labeled protein solution were injected onto an HPLC Gel filtration column, BIO-SIL SEC-250 (300x7.8 mm). The mobile phase solution used for all the HPLC experiments was sulfate-phosphate buffer at pH ~6.8. Na_2SO_4 (0.05 M) and NaH_2PO_4 (0.02 M) were dissolved in HPLC grade water and the pH was adjusted with 1 M sodium hydroxide. This solution was filtered and degassed through a 0.4 μ filter. The flow rate was fixed at 1 mL/min, and the chart speed was either 60 cm/h or 30 cm/h.

<u>Experiments</u>

A. Determination of the Retention Time of Human γ Globulin

A standard protein mixture, containing human γ globulin obtained from Bio-Rad, was

injected on the column. The column was washed with the same buffer solution as above for 30 min. Then 20 μL of 7.5 mg/mL IgG was injected. The shape of the peaks and the retention times were compared.

B. Effect of DMF and $CSCl_2$ on the Protein

25 μL of DMF was mixed with 0.5 mL of IgG and incubated for 1 hr (this solution serves as a control). 50 μL solution of $CSCl_2$ in $CHCl_3$ was evaporated under a stream of air and redissolved in 25 μL of DMF and incubated with 0.5 mL of IgG for 1 hr. 20 μL of each solution were injected on the HPLC column. The shape of the peaks and the retention times were compared with the chromatogram of the untreated IgG.

C. Determination of the Labeling Yield using HPLC

20 μL of the labeled protein (IgG) were injected on the HPLC column. The radioactivity associated with the protein peak was compared to the total radioactivity recovered.

D. Comparison Between the Yield Obtained from HPLC and G-75 Sephadex Open Column

The labeling yield of the IgG after 24 hr of incubation was determined by the HPLC and the open column method following the procedures described above. The % yield determined by the two methods was compared.

E. Stability Study of the Labeled Protein

Human γ globulin was labeled with the Tc-99m complex following the procedure described above and incubated at room temperature for 24 hrs. 20 μL aliquots were injected onto the HPLC column after 1 hr, 2 hr, 4 hr, and 24 hr of incubation.

F. Labeling of Antibody B72.3

The complexation and conjugation procedure was the same as described above for IgG. The same HPLC experiments done for the IgG were repeated with the antibody using ligand 1 only.

G. Determination of the Effect of the Labeling Technique on the Active Sites of the Antibody

Procedure:

1. Immobilization of the Mucin (antigen) to the activated sepharose was accomplished by stirring with the Mucin overnight at 4°C.
2. The excess ligand was removed by washing. The remaining active groups were blocked by stirring the sepharose with 1 M ethanolamine at room temp. for 2 hr.
3. The microcolumns were packed with the sepharose from above and equilibrated with PBS (phosphate buffered saline) + 1% BSA (bovine serum albumin).
4. A 50 μL aliquot of the sample was applied to duplicate columns and washed with

2x1 mL PBS + 1% BSA.

5. The columns and the wash were counted for radioactivity to determine the % binding.

III. Results

The complexation yield of the Tc complexes was determined by the extraction technique. The yield was close to 100% (95-97%) for both ligands **1** and **2**.

Figure 14 shows the HPLC elution profile for untreated IgG. The HPLC elution profile showed no effect of DMF and $CSCl_2$ on the protein. This experiment was designed to determine the extent of denaturing or aggregation that would be caused by these two agents during conjugation (Figs. 15 & 16).

The labeling yield of IgG using the Tc complex of ligand **1** as determined by HPLC was 70% after 1-2 hr of incubation. The yield was over 80% when determined by the open column method. This result suggests that some of the activity may have been loosely bound to the protein and was displaced during elution from the column (Fig. 17, P. 1).

The stability of the protein labeled with the Tc complex of ligand 1 was low. The activity associated with the protein was found to decrease with time (Fig. 17, P. 1-5), while the activity associated with pertechnetate or unconjugated complex was found to increase. Characterization of the non-protein peak showed that the activity was in the form of pertechnetate not unconjugated complex. This result suggests that the ligand is releasing the Tc as it oxidizes to pertechnetate while the bond between the protein and the ligand stays intact.

Ligand 2 gave better results. No dissociation was observed after 16 hr of incubation (Fig. 18) as determined by HPLC. The conjugation yield of this ligand as determined by the open column technique is in good agreement with that obtained using HPLC. This indicates that all radioactivity is rather tightly bound. However, the conjugation yield was only in the range of 60-65% (65%); more work needs to be done to improve the conjugation yield using this ligand.

B72.3 was studied with only the Tc complex of ligand **1**. The results were similar to those obtained with IgG. Good conjugation yields were obtained, but the conjugate was not very stable over 24 hr of incubation.

HPLC studies of the protein labeled with rhodium complexes have not yet been accomplished. The extraction of the rhodium complex into $CHCl_3$ was low hence the amount of activity was too low to be detected. Work is now directed toward improvement of the extraction of the complexes of ligands **1** and **2** or to obtaining a better method of purification. The immunoreactivity results are shown in the following table. The purified protein was obtained by purifying the labeled protein using the open column method and using the protein fraction. The mixture refers to the unpurified labeled protein. After purification about 90% of the antibody retained its radioactivity after labeling with the Tc complex, and 75% when labeled with rhodium

Fig. 14 Sample: Untreated IgG. Mobile phase 0.05M Na_2SO_4 -0.02M NaH_2PO_4 pH [illegible] speed 60 cm/h. Flow rate 1mL/min. 0.16 AUFS.

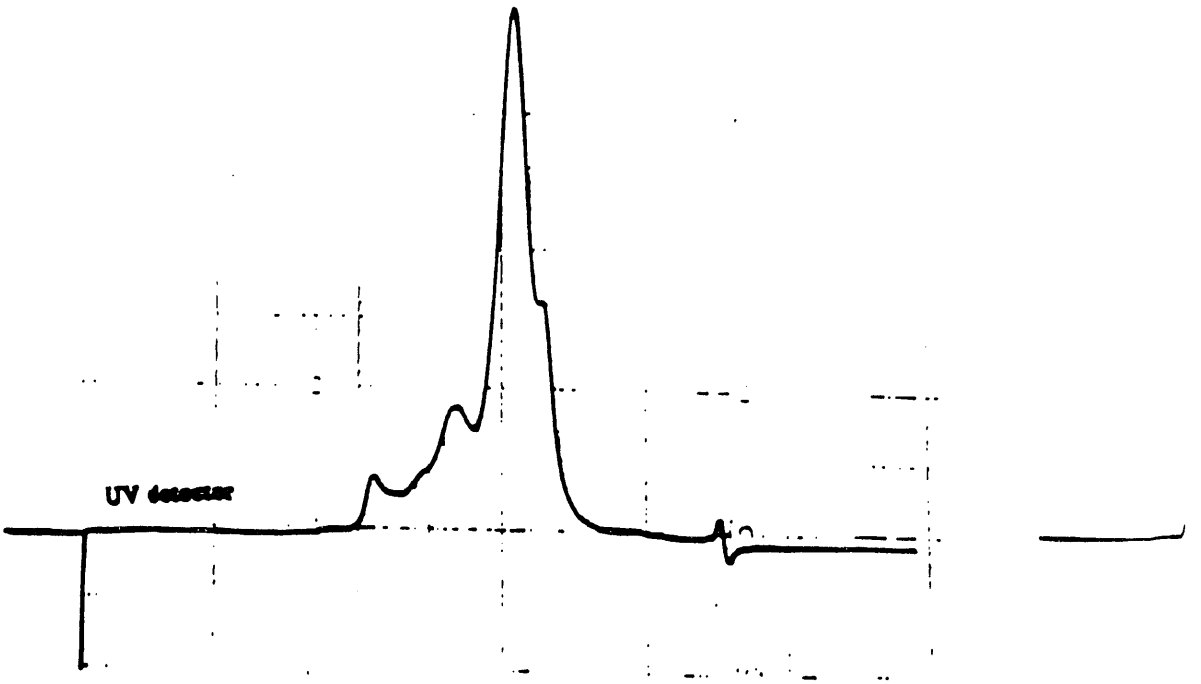


Fig. 15 Sample: IgG+25 µL DMF. Mobile phase 0.05M Na_2SO_4 -0.02M NaH_2PO_4 pH 6.8. Chart speed 60 cm/h. Flow rate 1mL/min. 0.16 AUFS.

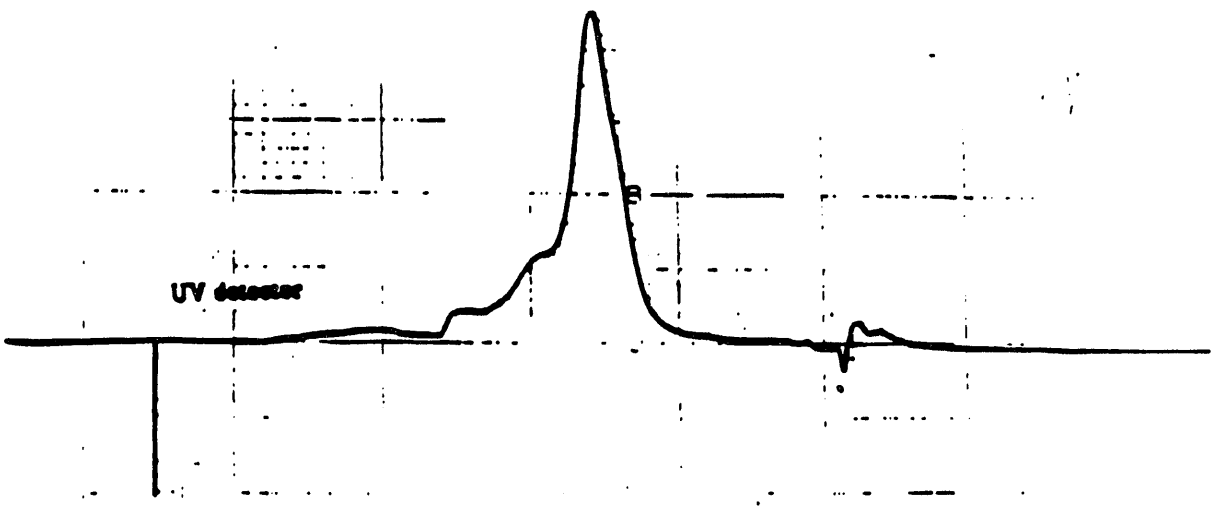


Fig. 16 Sample: IgG+DMF+$CSCL_2$. Mobile phase 0.05M Na_2SO_4 -0.02M NaH_2PO_4 pH 6.8. Chart speed 60 cm/h. Flow rate 1mL/min. 0.16 AUFS.

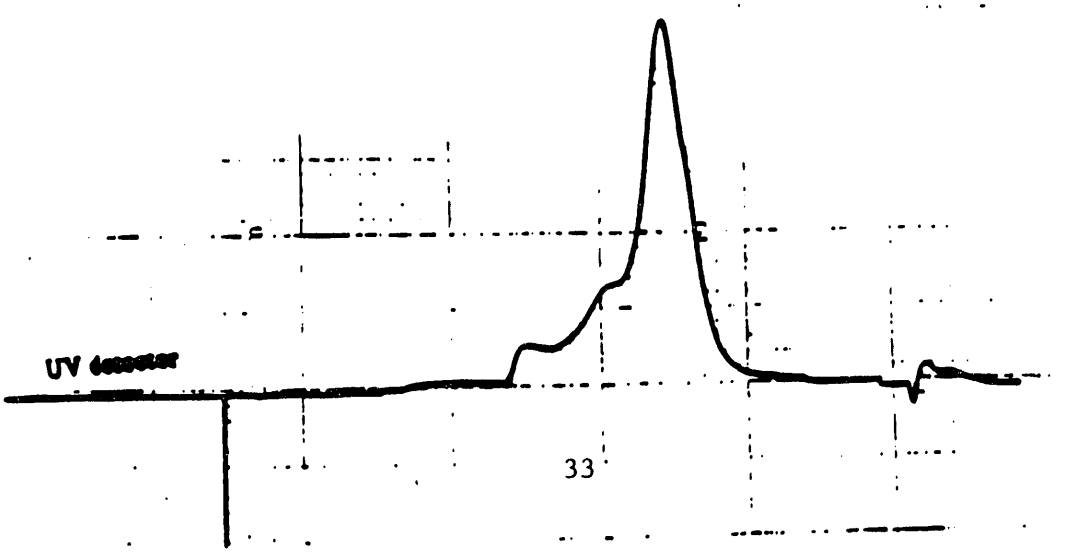


Fig. 17-P1

Sample Labeled IgG with Tc 99m using ligand 1, 50 min incubation. Mobile phase 0.05M Na_2SO_4 0.02M NaH_2PO_4 pH 6.8. Chart speed 60 cm/h. Flow rate 1ml./min 0.16 AUFS

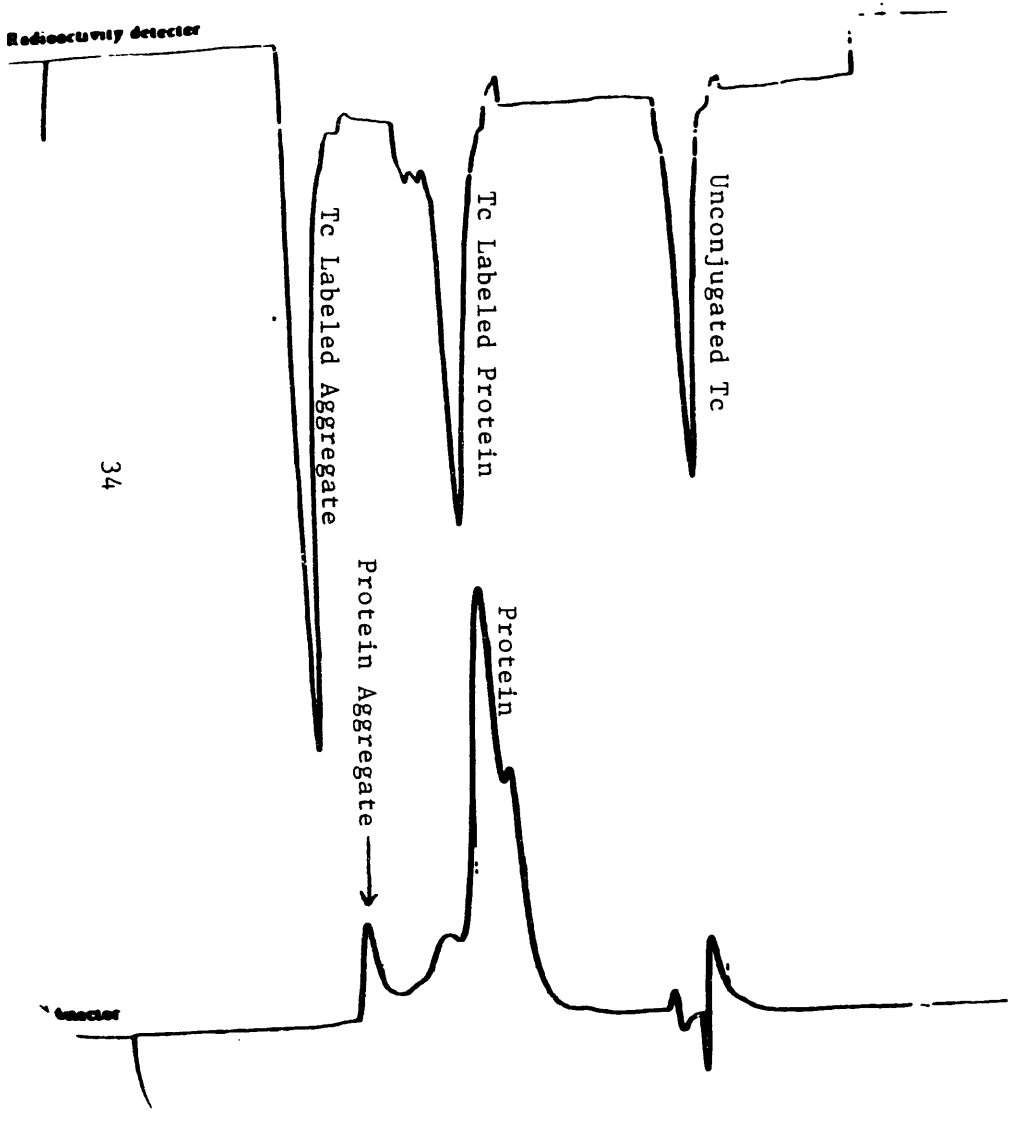


Fig. 17-P2

Sample Labeled IgG with Tc 99m using ligand 1, 120 min incubation. Mobile phase 0.05M Na_2SO_4 0.02M NaH_2PO_4 pH 6.8. Chart speed 60 cm/h. Flow rate 1ml./min. 0.16 AUFS

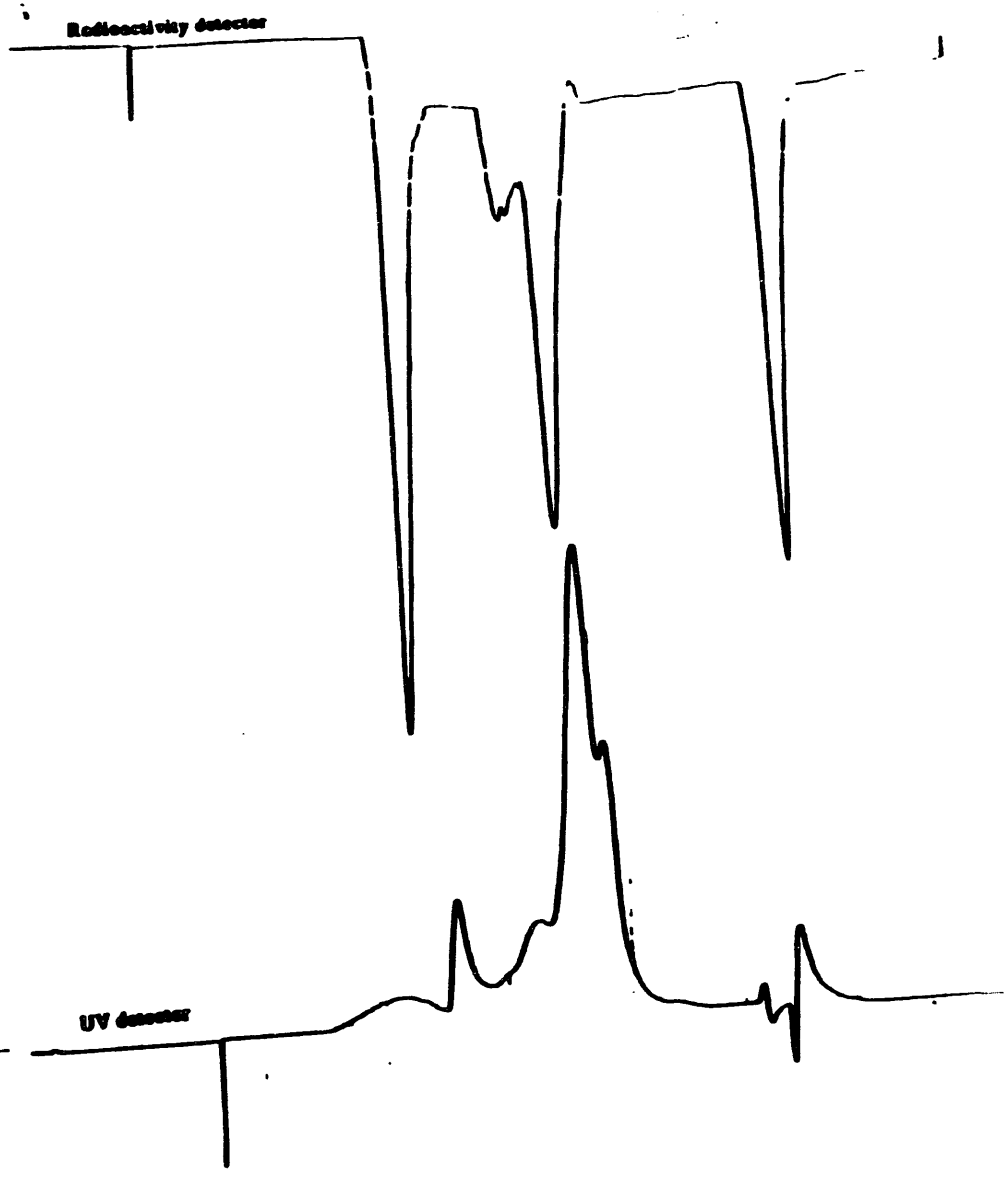


Fig. 17-P3

[illegible] Chart speed 60 cm/h. Flow rate 1mL/min. 0.16 AUFS

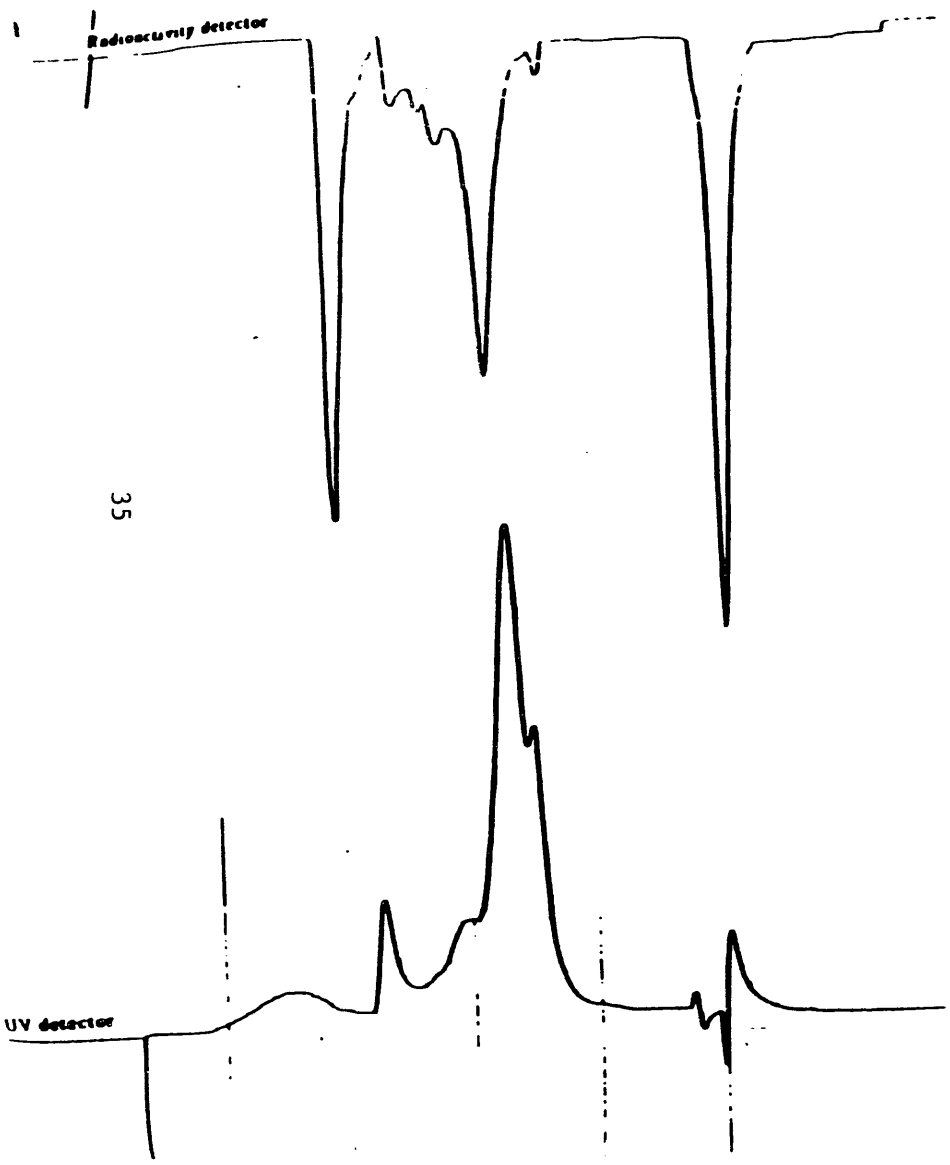


Fig. 17-P4

Sample: Labeled IgG with Tc 99m using ligand 1, 10h incubation time. Mobile phase 0.05M Na_2SO_4 0.02M NaH_2PO_4 pH 6.8. Chart speed 60 cm/h. Flow rate 1mL/min 0.16 AUFS

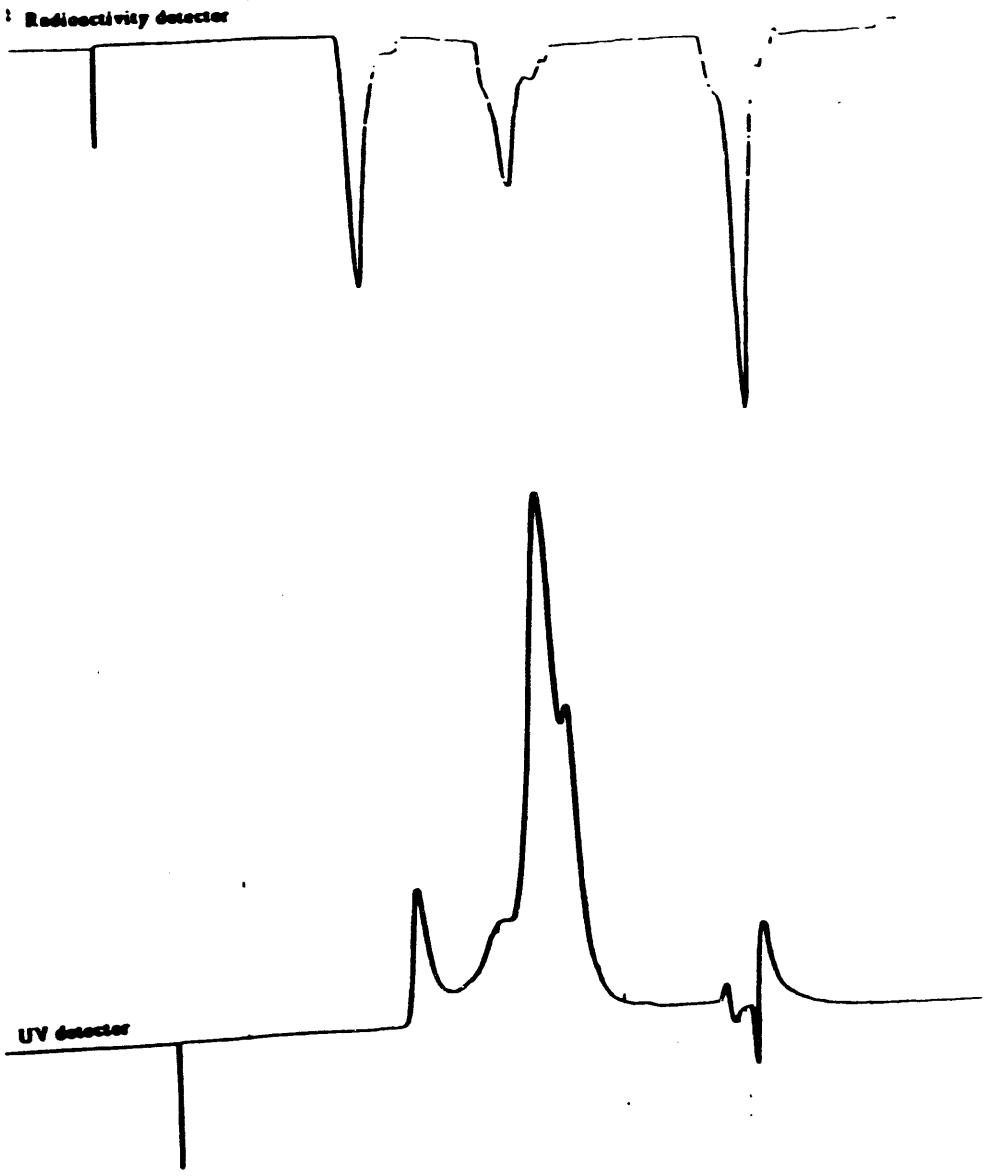


Fig. 17-P5

Sample: Labeled IgG with Tc-99m using ligand 1, 24h incubation time. Mobile phase 0.05M Na_2SO_4 -0.02M NaH_2PO_4 pH 6.8. Chart speed 60 cm/h. Flow rate 1mL/min. 0.16 AUFS.

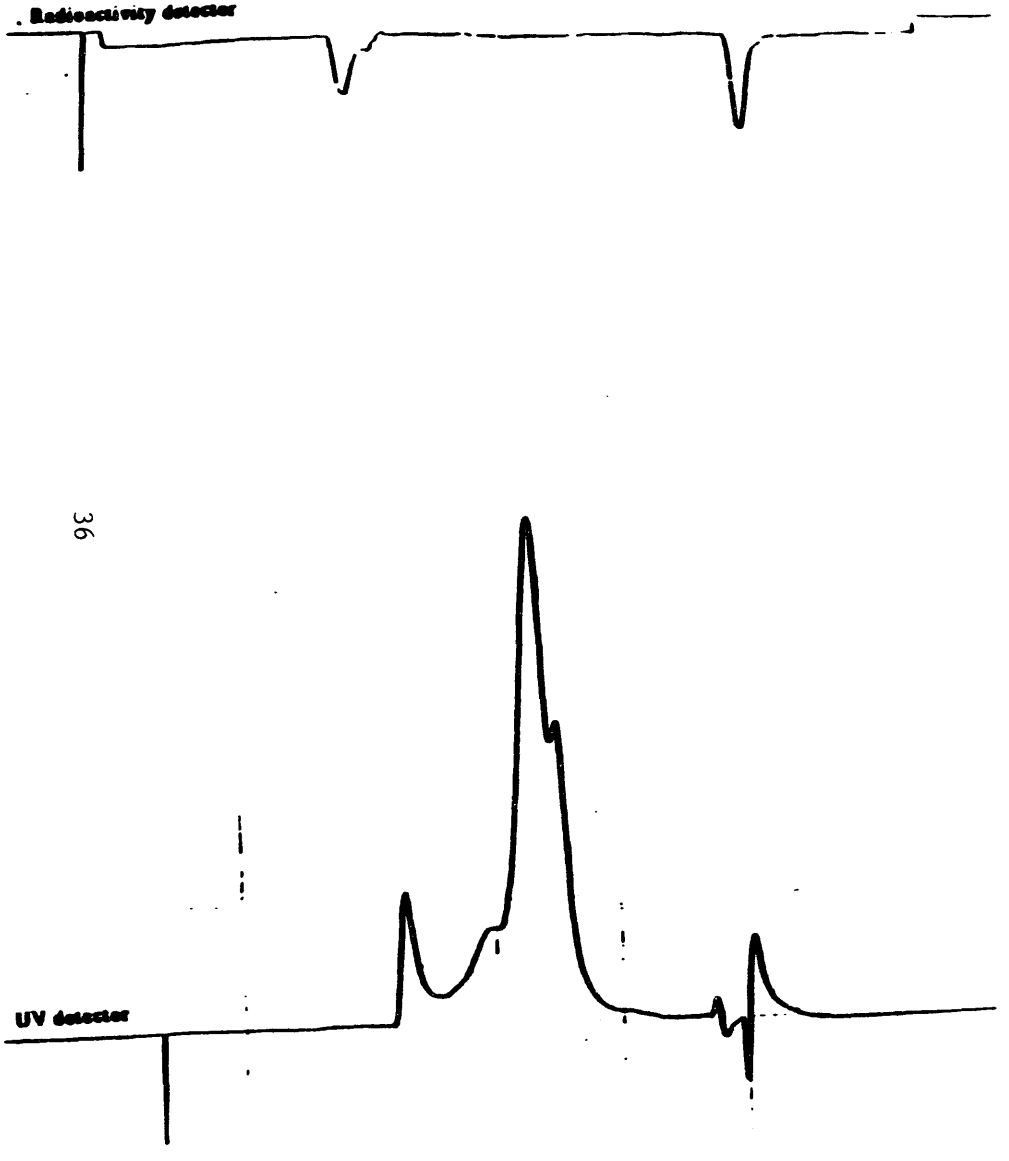
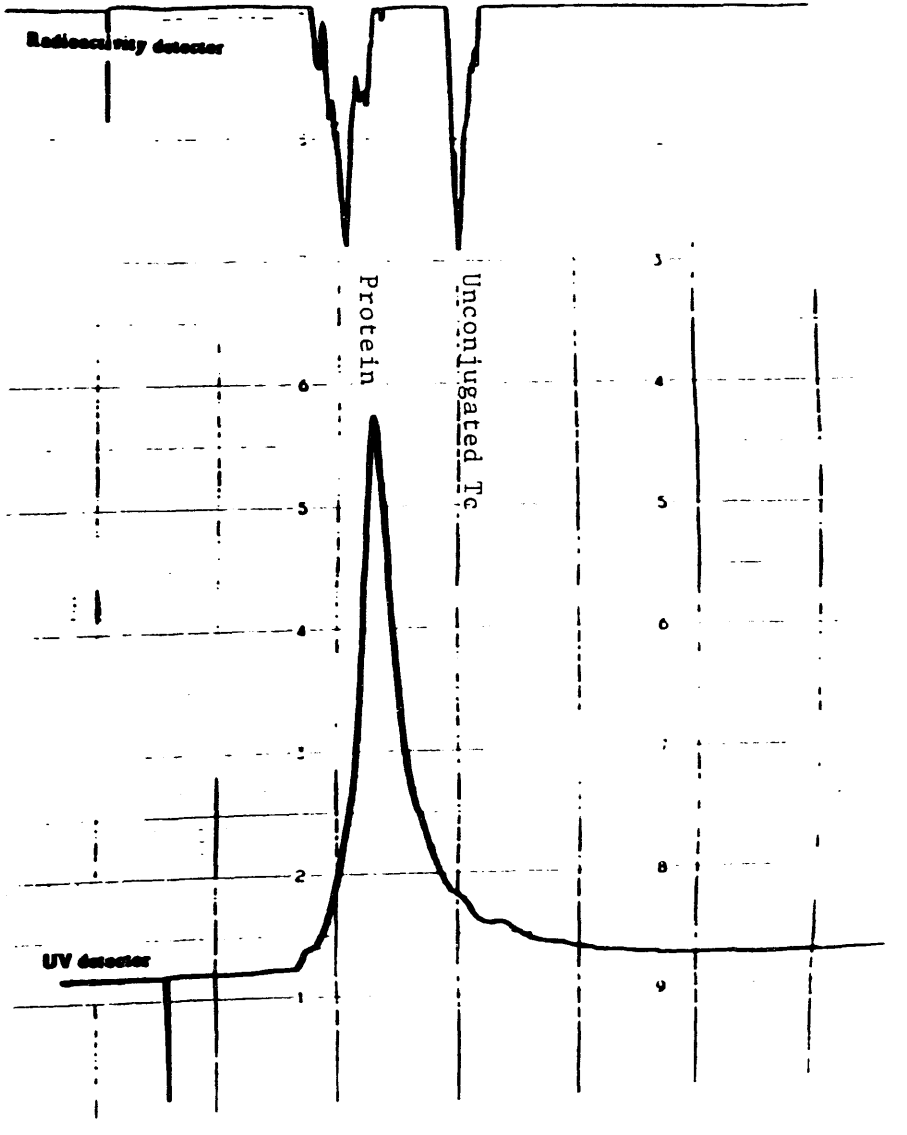


Fig. 18

Sample: Labeled IgG with Tc-99m using ligand 2, 16h incubation time. Mobile phase 0.05M Na_2SO_4 -0.02M NaH_2PO_4 pH 6.8. Chart speed 30 cm/h. Flow rate 1ml/min. 0.16 AUFS.

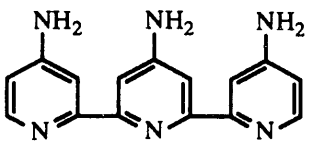


complex.

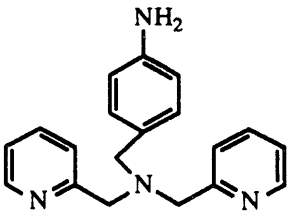
Sample	% Binding	Ave.
Tc		
Labeled AB mixture (Unpurified)	57.5, 59.8	58
Purified	91.1, 89.3, 84, 88	88.1
Complex Only	10, 12.8	11.4
Rh-105		
Mixture	50, 50	50
Purified	71, 75	73

6. Progress on Ligand Systems for Au(III)

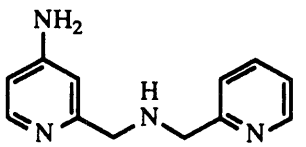
Studies to date have emphasized multidentate nitrogen donor ligands because of the previously reported stable complexation of Au(III) with terpyridyl at near neutral pH reported by Holmes and Lippard (10). Thus we have been examining the following possible ligand systems:



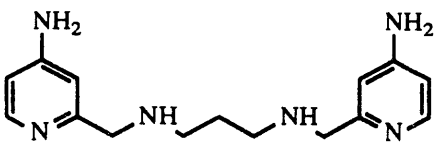
I



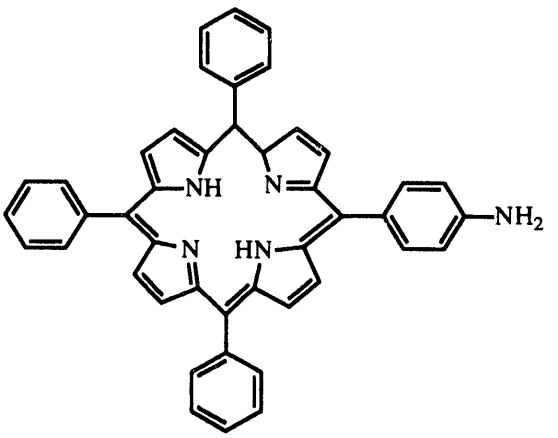
II



III



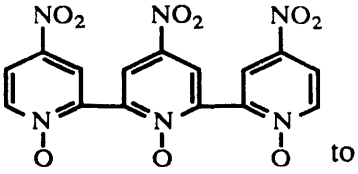
IV



V

Results to date are presented below for each case.

Ligand I

This ligand was chosen because of the known stability of the unsubstituted terpyridyl complex of Au(III) (10) and because its synthesis has been reported earlier (11). We have encountered some difficulty in the three step procedure for ligand synthesis but have now successfully completed the first two steps. We have obtained purified products at both points, and these have been characterized by NMR and melting points. We are currently determining the best approach to the final step of reduction of  to produce ligand I. This project is part of the thesis research of Mr. David Klug.

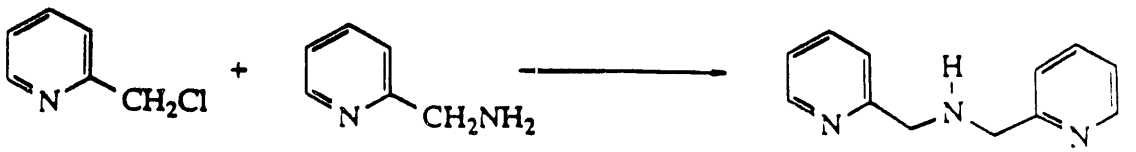
Ligands II, III and IV

Ligand synthesis schemes are outlined on the following pages. They include specific approaches to ligands II and III as well as successful methods for the unsubstituted analogs. The amino-substituted ligand IV has not yet been synthesized. The syntheses described in the schemes have been completed except for the final stages of 4(ii).

Synthesis Schemes for Ligands II and III

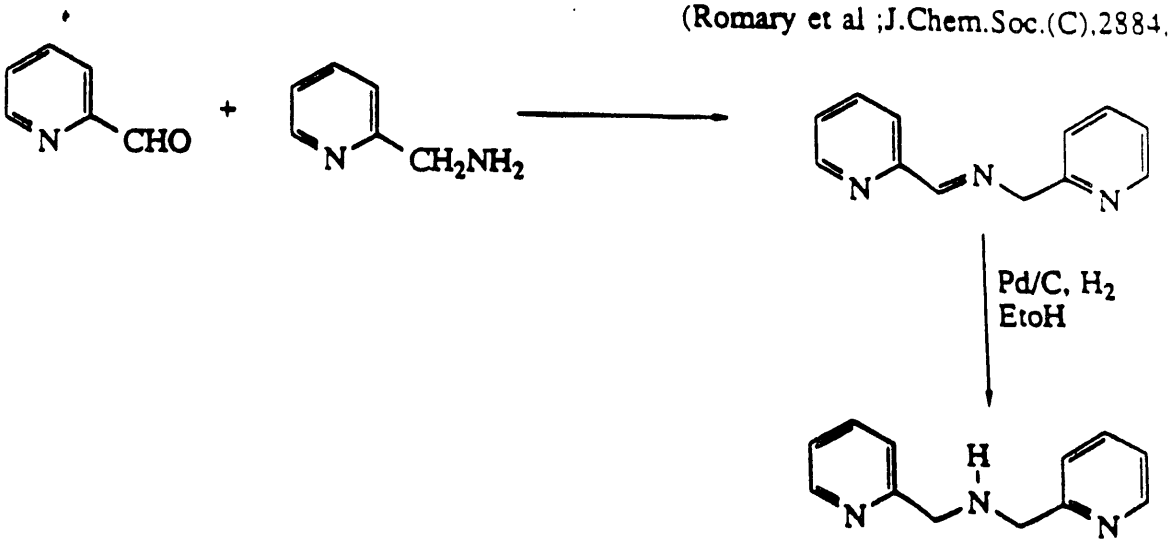
1)Dipicolylamine

(a)



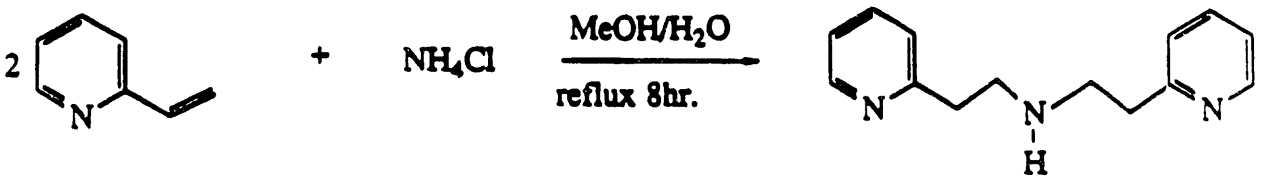
(Romary et al ;J.Chem.Soc.(C),2884,1968)

(b)



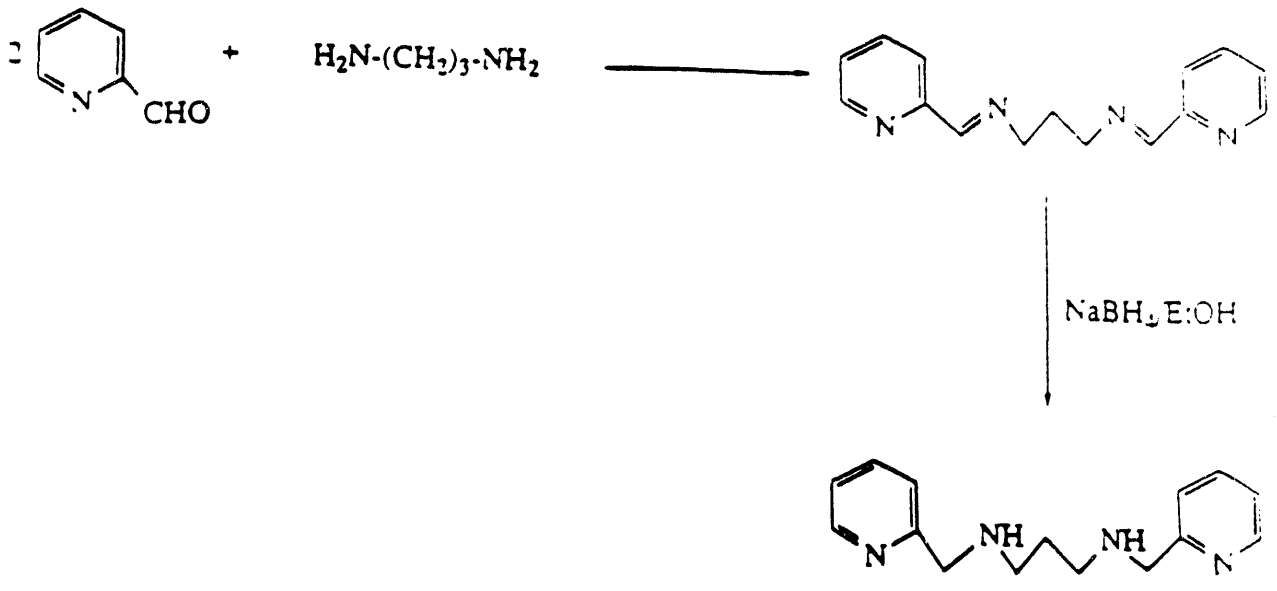
(Gruenwedel ;Inorg.Chem. 7,495,1968)

2) Bis- (2-pyridylethylamine)



(Brady,et al;J.O.C.,26,4758,1961)

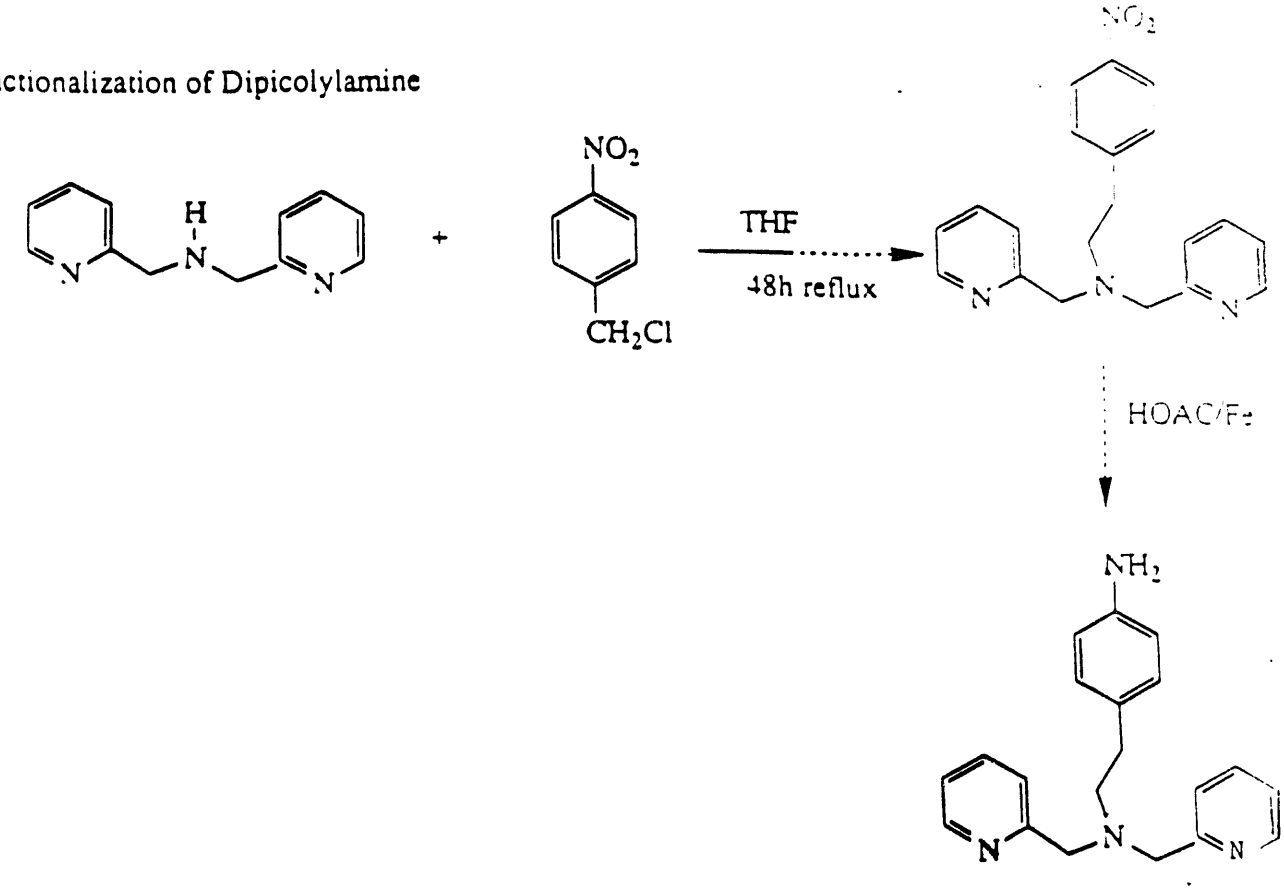
3) 1,7-bis(2-pyridyl)-2,6-diazaheptane



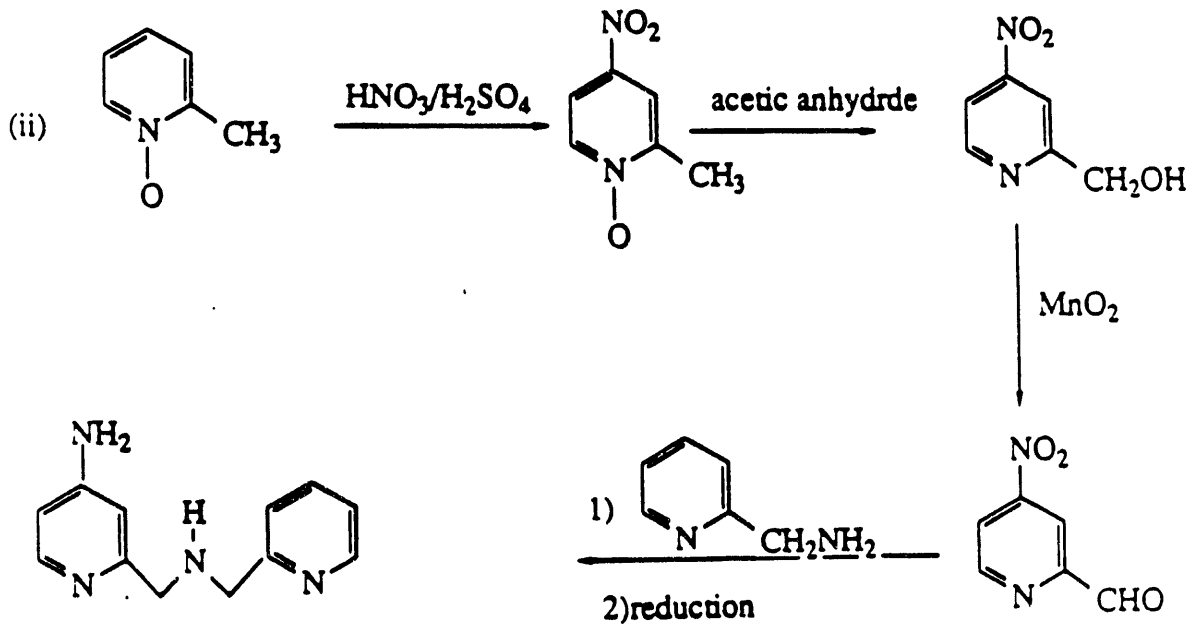
(Newkome et al; Inorg.Chem. 24, 1001, 198'

4) Functionalization of Dipicolylamine

(i)



(ii)



Crystal structures of both the Rh(III) and Au(III) complexes (Figures 19 and 20) of the unsubstituted dipicolylamine ligand show the expected bonding behavior. If similar geometries are obtained with ligands II and III as expected, the aromatic amine functional group should be in a favorable steric orientation for protein conjugation.

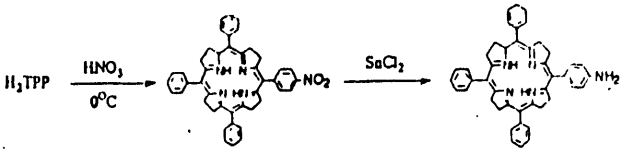
Initial radiochemical studies with ligand II and dipicolylamine (DPA) have been done with Rh-105, Pd-109, and Au-198. All of these studies are promising. DPA gave 95% complexation with Rh-105 while ligand II gave 88%. Preliminary studies of conjugation of Rh-105 to HSA with ligand II using thiophosgene are promising but incomplete.

Ligand II, DPA, and the unsubstituted parent of ligand IV all gave promising complexation with Pd-109 as determined at radiochemical levels by paper electrophoresis or paper chromatography. The complexation yields ranged from 79-92% with ligand II giving 92%. In addition a preliminary electrophoresis study with Au-198 shows the formation of a neutral Au complex. This is not expected and may be a result of redox decomposition. Further study with ion exchange chromatography will be done.

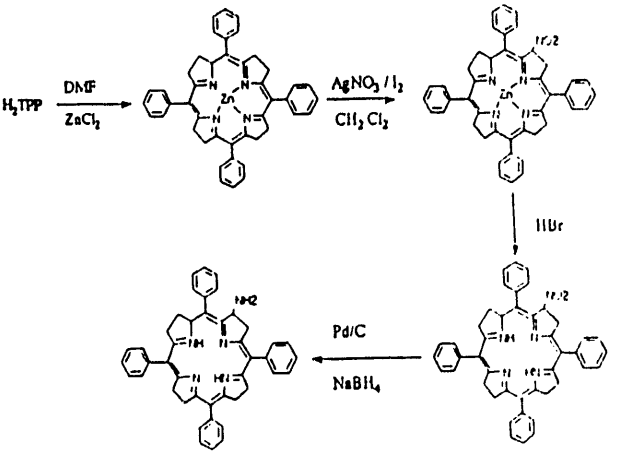
More quantitative studies of the complexation at the radiopharmaceutical level and conjugation to proteins is underway as part of the Ph.D. thesis work of Mr. Nihal Weerasinghe.

Ligand V

This ligand as well as a related 2-amino substituted isomer have been successfully synthesized (see reaction schemes below).



Basic Synthesis of 5(4 amino) 10, 15,20 Tetraphenylporphyrin



Basic Synthesis for 2-amino 5,10,15,20 Tetraphenylporphyrin

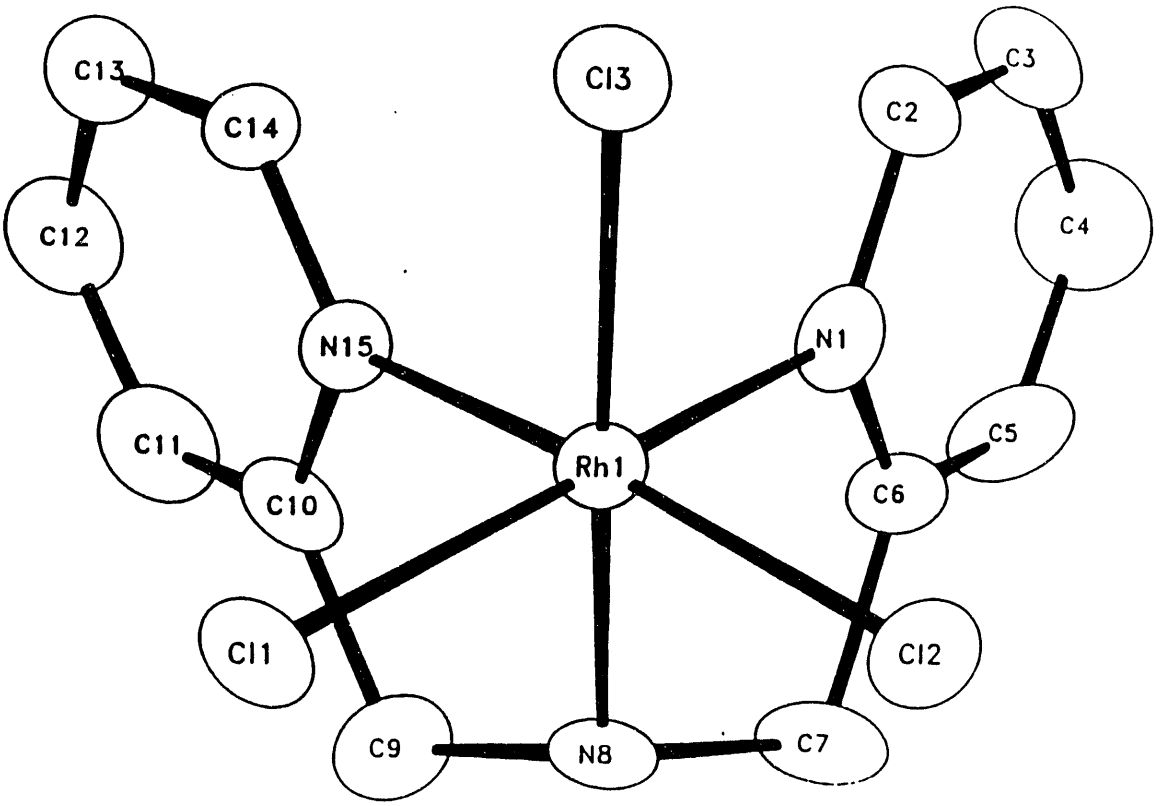


Figure 19

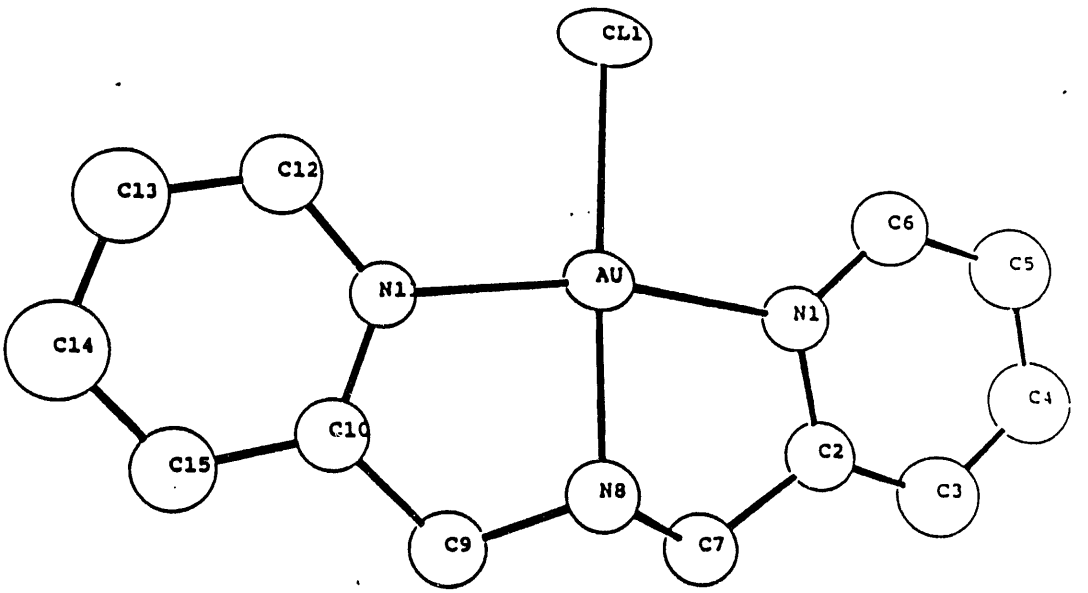


Fig. 20 X-ray structure of Au(III) dipicolylamine complex

In the latter scheme the Zn TTP intermediate was isolated, and its x-ray structure was completed (Figure 21). Cold chemistry with Pd(II) and Rh(III) indicate complexation with ligand V (color changes as well as NMR and IR characterization). Radiochemical studies with Pd-109 and Rh-105 were performed (100:1 ligand/metal ratio). The complexation was followed by paper electrophoresis, paper and thin layer chromatography, solvent extraction, and for Rh(III) MgO adsorption. For both Pd(II) and Rh(III) the complexation yields were disappointing (32 and 33% respectively). Although we will examine the complexation of Au(III) (Au-198) with these ligands at radiochemical levels we anticipate similar results and have already made plans to change to a new ligand system (Ph.D. thesis project of Ms. Pat Droege). The new ligands systems will be appropriately functionalized derivatives of 2-hydroxyacetophenone. Preliminary work with this system is very promising.

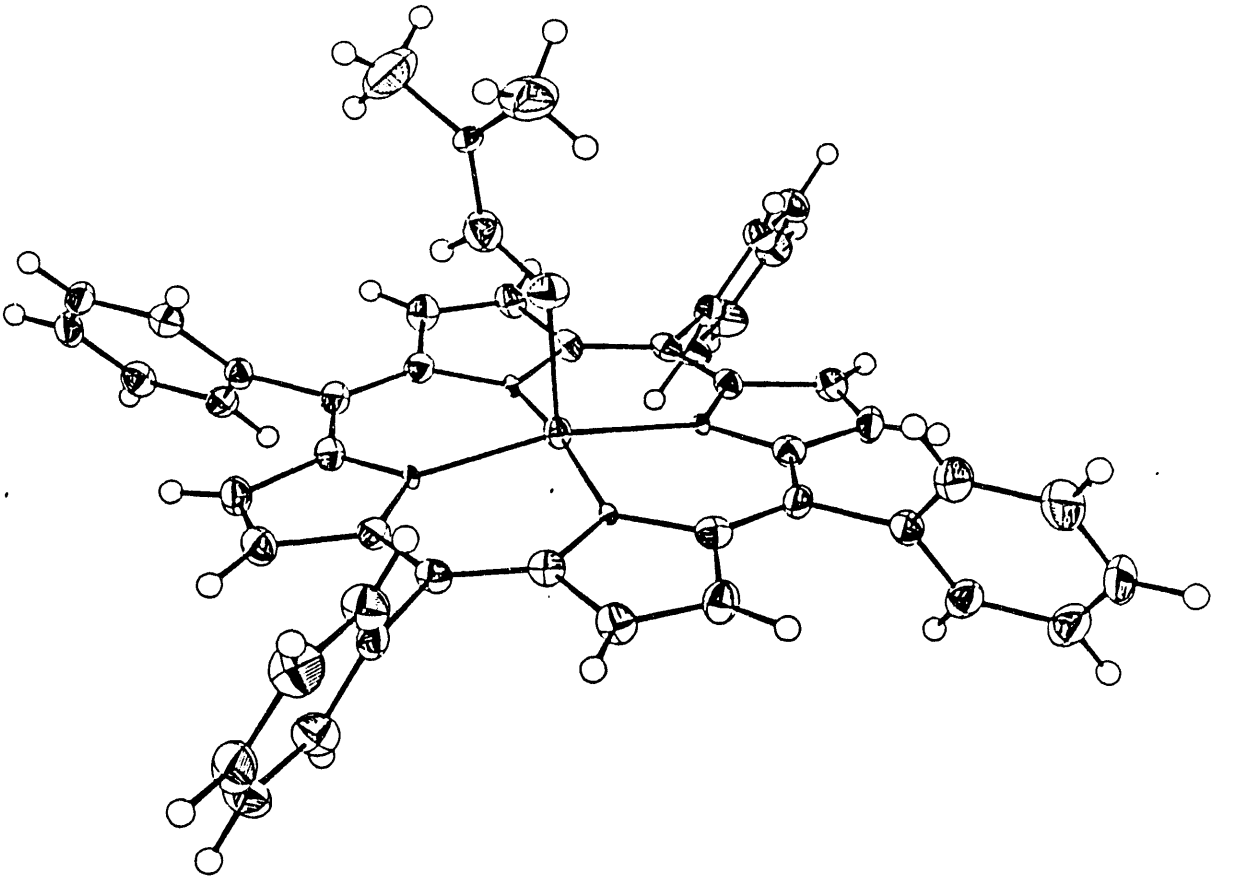


Fig. 21 ZnTPP

Brief Summary of Research Progress

1. Production of Radioisotopes

With the highest flux research reactor at any U.S. University, the MURR, we at the University of Missouri-Columbia have ready access to high specific activity reactor-produced radioisotopes. Many of the radioisotopes used in our research were produced by direct neutron capture on stable isotopes of the same element (i.e. Au-197 (n,γ) Au-198). However, Rh-105 is produced by neutron capture on Ru-104 to produce Ru-105, which decays with a 4.4 hour half-life to Rh-105 ($t_{1/2}$=35.5 hours). A method was developed to remove the Ru target material yelding very high specific activity (no carrier) Rh-105 (1). The Ru metal target is first oxidized to RuO_4 by bubbling Cl_2 through a NaOH solution of the target. The volatile RuO_4 is distilled off leaving the Rh-105 in solution. Excess oxidant is decomposed by adding acid and heating. The resulting solution is then used as the source of Rh-105 for our studies. This procedure has been used to prepare >100 mCi of Rh-105 per process on a weekly basis.

2. Developed Several Bifunctional Chelating Ligand Systems which gave Successful *in vitro* Labeling of Rh(III) and Pd(II)

Several ligand systems based on amine-oximes (bi-, tri- and tetradentate), tridentate amines, amine-pyrroles (penta- and hexadentate) and tetradentate imine- and amine-phenols were synthesized and investigated as potential bifunctional chelates for Rh(III) and Pd(II). The benzyl-amine functionalized derivatives of the amine-oxime and amine-phenol ligands proved to be the most successful bifunctional chelating ligands. The ^{105}Rh(III) complexes formed with the pentadentate amine-phenol ligand (Figure 6) and the tri- and tetradentate amine-oxime ligands gave high complexation yields and reasonably good protein conjugation yields. The pentadentate amine-phenol ligand also gave satisfactory complexation and protein conjugation yields with ^{109}Pd(II). The *in vitro* stability studies of these four complexes under biological conditions (described in the proposal) will be investigated to screen these complexes as potential candidates for *in vivo* rat biodistribution studies.

3. Macroscopically Characterized Many of the Ligands and Complexes by Methods including IR, NMR, Elemental Analyses, and X-ray Structure Determination

The ligand systems described in the text have been characterized by IR, NMR and elemental analyses, as have been many of their metal complexes. The x-ray structural determinations of the Rh(III) complexes of the benzyl analogs of the bidentate amine-oxime ligand and the tridentate amine ligand (Figure 2) show that an appropriately functionalized benzyl group

would be oriented such that it would be available for protein conjugation. This has indeed been verified on the radiochemical level where protein coupling yields of up to 90% were observed with the ^{105}Rh complex with ligand I (p.1) and up to 75% with the ^{105}Rh complex with ligand III (p. 1).

Approximately fifteen x-ray structure determinations have been performed on metal complexes of new ligands developed in this work.

4. Made Progress on Developing Ligand Systems for use with Au(III)

Five ligand systems containing amine, pyridine and/or pyrrole nitrogen donors have been selected for complexation with Au(III). The dipicolylamine ligand has been synthesized and the Au(III) complex prepared and characterized by its x-ray crystal structure. The tetrapyrrole porphyrin ligand has also been synthesized and structurally characterized as its zinc complex. Preliminary studies using this ligand to complex Rh(III) and Pd(II) have been disappointing. Although we plan to investigate this ligand system with Au(III), we anticipate similar results. Three other ligand systems are in various stages of preparation. As these ligands are prepared, their chemistry with Au(III) will be investigated both on the macroscopic and radiochemical levels.

5. Protein Assay Method

Since gel permeation chromatography was used for our protein binding studies, we needed a protein assay method to determine total protein recovery. Both the Lowry and the Bio-Rad assay methods were examined. We discovered that our ligands, which contained phenol groups, significantly interfered with the Lowry method. This is not unexpected since one of the reagents used in the Lowry method is a phenol. The Lowry method was also affected by the buffer used: for example the Tris buffer showed significant interference. The Bio-Rad Protein assay was not affected by these factors and was much easier to perform than the Lowry method. The Bio-Rad assay was also better suited to analysis of small samples, requiring only 0.2 mL of a single reagent compared to larger quantities of two reagents for the Lowry method. We concluded that the Bio-Rad protein assay method was superior to the Lowry method for our purposes; it is the method we currently use.

6. HPLC Analysis of Protein/Complex Conjugates

Although Sephadex G75 gel filtration chromatography gave us a crude indication of our protein labeling efficiency, it could not distinguish between protein and protein aggregates. A High Performance Liquid Chromatography (HPLC) method was developed which effectively separated the aggregated and unaggregated protein. A BIO-SIL SEC-250 (300x7.8 mm) gel filtration column with a pH 6.8 0.05 M Na_2SO_4/0.02 M NaH_2PO_4 buffer as the mobile phase gave

the desired separation. Both a UV detector and a radioactivity detector were used. The UV detector identified the protein and protein aggregates while the radioactivity detector identified the radioisotope. By comparing the traces from the two detectors, we could determine which, if any, of the protein components were labeled and how much of the radioisotope was associated with each component. This analytical method also made it possible to examine how the conjugation procedure might affect the protein with respect to aggregation. Using this HPLC method, we determined that the DMF and thiophosgene used in the conjugation procedure did not cause aggregation of the protein.

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TRAINING OF GRADUATE STUDENTS AND POST-DOCTORALS

Graduate Students Supported in Part by this Grant

Several graduate students have participated in this program. Most have received only a year or less of support which has lead to the involvement of more students in the overall project. Data below show (name, year of participation in grant, degree and year, and present occupation)

Tonya Lynde-Kernell, 1986, M.S. 1986, chemist at General Dynamics, Dallas, TX

Brent Grazman, 1986, M.S. 1986, Nat'l Bureau of Standards, Gaithersburg, MD

Yai-Kai Wong, 1986-87, M.S. 1987, chemist at MA General Hospital in Urology

Craig Schultz, 1987, completed thesis requirements for M.S. , chemist at Dow Chemical, Freeport, TX

Renfeng Wang, 1986, Ph.D. 1990, Post-Doc at Univ. of WI-Madison

Guler Ergun-Efe, 1987-89, Ph.D. 1989, teaching at Southwest Louisiana

Khaled Misellati, 1988-91, Ph.D. 1989, accepted a position in Australia

Tim Kilcoin, 1986-91, Ph.D. expected 1992, presently employed at ABC Labs

Lixin Lang, 1987-91, Ph.D. expected 1992

Nihal Weerasinghe, 1989- , Ph.D. candidate

Pat Droege, 1990- , Ph.D. candidate

David Klug, 1991- , Ph.D. candidate

Postdoctoral Associates

Christy S. John, Ph.D. UMSL 1987, employed June 1987-June 1988, now heading the radiopharmaceutical program at George Washington University Medical Center.

Jem Mau Lo, Visiting professor in radiochemistry from National Tsing Hua University, Hsinchu, Taiwan, August 1987-July 1988, returned home university to continue expanding radiopharmaceutical research at that institution.

M.R.A. Pillai, Visiting Scientist, September 1987-September 1989. His present occupation is doing radioimmunochemistry at Bhabha Atomic Research Center, India.

PUBLICATIONS

In the list of publications the following initials will be used:

TLK=Tonya Lynde-Kernell
DET=David E. Troutner
EOS=Elmer O. Schlemper
CJ=Christy John
MRAP=M.R.A. Pillai
JML=Jem-Mau Lo
GEE=Guler Ergun-Efe
BG=Brent Grazman

1. "Chemical and Physical Properties of Radionuclides", DET, *Nucl. Med. Biol.*, **1987**, *14*, 171.
2. "Preparation, Characterization and Crystal Structures of Two Amine-Oxime Rhodium Complexes", TLK and EOS, *J. Coord. Chem.*, **1988**, *16*, 347.
3. "*Trans*-Dichlorobis(ethylenediamine)rhodium(III)-Nitrate and Hydrogen Oxalate Salts", TLK and EOS, *Acta Cryst.*, **1988**, *C44*, 31.
4. "^{105}Rh as a Potential Radiotherapeutic Agent", BG and DET, *Appl. Radiat. Isot.*, **1988**, *39*, 257.
5. "Labeling of Proteins with ^{105}Rh", CJ, MRAP, JML, DET, *Appl. Radiat. Isot.*, **1989**, *40*, 701.
6. "Labeling of Human Serum Albumin with ^{105}Rh-cysteine Complexes", JML, MRAP, CJ, DET, *Appl. Radiat. Isot.*, **1990**, *41*, 63.
7. "Radiochemical Purity Evaluation of Rhodium-105 Complexes by Magnesium Oxide", JML, MRAP, CJ, DET, *Appl. Radiat. Isot.*, **1990**, *41*, 103.
8. Labeling of Hematoporphyrin with ^{105}Rh and Binding Studies with Human Gamma Globulin", MRAP, JML, DET, *Appl. Radiat. Isot.*, **1990**, *41*, 69.
9. "Labeling of Proteins using (^{105}Rh)Rh-4-(p-aminobenzyl)-diethylenetriamine", MRAP, JML, CJ, DET, *Nucl. Med. and Biol.*, **1990**, *17*, 419-26.
10. "Pentadentate Amine Phenol Complexes of ^{99m}Tc", DET, MRAP, CJ, JML and K. Misellati, *Proceedings of Third International Symposium on Tc in Chemistry and Nuclear Medicine*, Padova, Italy, **1990**, *3*, 585.
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13. "Labeling of Human Gamma Globulin with (^{105}Rh) Rhodium using a New Pentadentate Bifunctional Ligand", MRAP, CJ, DET, *Bioconjugate Chem.*, **1990**, *1*, 191-97.
14. "Rhodium Complexes of Some Bidentate Secondary Amine-Oxime Ligands and Application to Labeling of Proteins", GEE, MRAP, EOS, DET, *Polyhedron*, **1991**, 1611-16.
15. "Synthesis of an Ether Oxime Ligand and its Rh(III) Complex and X-ray Structure of the Rh(III) Complex: Unique Rh(III) Bonding to a Simple Ether", GEE and EOS, *Polyhedron*, **1991**, 1617-24.

MANUSCRIPTS SUBMITTED

1. "Technetium Complexes of Pentadentate Amine Phenol Ligands", MRAP, CJ, JML, M. Corlija, DET, *Appl. Radiat. Isot.*
2. "Rh(III) Complexes of New N-Substituted α-Amine Oxime Ligands: Synthesis and Crystal Structures of [Rh(phenAO)$_2$Cl$_2$], [Rh(phetAO)$_2$Cl$_2$], [Rh(acetanilidoAO)$_2$Cl$_2$] and [Rh(N-MethylacetanilidoAO)$_2$Cl$_2$]", GEE and EOS, *Polyhedron.*

MANUSCRIPTS IN PREPARATION

1. "Pyrrole Derivatives of Triethylenediamine as Bifunctional Chelating Ligands", K. Misellati and DET, *Appl. Radiat. Isot.*
2. "Derivatives of 3,4-Diaminobenzoic Acid as Bifunctional Chelating Agents", Lixin Lang and DET, *Appl. Radiat. Isot.*
3. "Complexes of New Amine-Nitrogen Substituted α-Amine Oxime Ligands (p-Aminobenzyl, p-nitrobenzyl, and p-aminophenylethyl substituents): X-ray Structures of p-AminobenzylAO Dihydrochloride and [Ni(aminobenzAO)$_2$]ClO$_4$", GEE and EOS, *Polyhedron.*
4. 2-3 papers from the Ph.D. thesis of Tim Kilcoin on tri-and tetradentate amine oxime complexes (functionalized and unfunctionalized) (macroscopic and radiochemical studies).

PRESENTATIONS

<u>Nat'l Meeting, Am.Chem. Soc., Los Angeles, September, 1988</u>

1. "Rh(III) and other Metal Ion Complexes of Nitrogen Functionalized *alpha*-Amine-Oxime Ligands", EOS, GEE, and T. Kilcoin.

<u>Nat'l Meeting, Soc. Nucl. Med., San Francisco, June, 1988</u>

2. "Practical Considerations for Identifying Radionuclides for Therapeutic Applications", DET.

3. "Labeling of Proteins by Triamine Complexes of Rh(III)", CJ, MRAP, JML and DET.

Seventh Int. Symp. on Radiopharm. Chem., Groningen, July, 1988

4. "Production of Rh-105 by the Szilard-Chalmers Process with ruthenium acetylacetonate", Y.K. Wong, A.R. Ketring, JML, and DET.

Fourth Asia and Oceania Congress of Nuclear Medicine, Taipei, November, 1988

5. Radiochemical Priority Evaluation of Rh-105 Complexes by Magnesium Oxide", JML, MRAP, CJ and DET.

6. "Labeling of Human Serum Albumin with Rh-105 using Cysteine as a Bifunctional Chelating Agent", JML, MRAP, CJ and DET.

Society of Nuclear Medicine, Annual Meeting, St. Louis, June, 1989

7. "Labeling of Human IgG with Rh-105 Complexes derived from diethylenetriamine", MRAP, CJ, JML and DET.

8. "Synthesis, Characterization, and X-ray Structures of Amine Phenol Complexes of Tc(V)", CJ, MRAP, JML, EOS and DET.

9. "Radiochemical Studies of Tc-99m Complexes of Tetradentate Amine Phenols", MRAP, CJ, JML and DET.

10. "Evaluation of Tc-99m-tetradentate Amine Phenols as Potential Cell Labeling Radiopharmaceuticals", M. Corlija, MRAP, CJ, JML, Det, T.J. Hoffman, W.A. Volkert and R.A. Holmes.

Third Int. Symp. on Tc Chemistry and Nuclear Medicine, Padova, italy, September, 1989

11. "Pentadentate Amine Phenol Complexes of Tc-99m", DET, MRAP, CJ, JML and K. Misellati.

American Chemical Society, Midwest Regional Meeting, St. Louis, November, 1989

12. "Synthesis, Characterization, and Structure of Some New Rhodium(III) Complexes as Potential Radiopharmaceuticals", T. Kilcoin and EOS.

American Crystallographic Association, Nat'l Meeting, Seattle, July, 1989

13. "Structure of Some Tc(V) and Pd(II) Complexes of Reduced Tetradentate Ligands Derived from Salicylaldehyde and Diamines", MRAP, CJ, JML, EOS and DET.

7th Annual Missouri Inorganic Day, St. Louis, April, 1990

14. "Development of Bifunctional Ligands and their Rh(III) Complexes for Potential Radiotherapy Kilcoin and EOS.

ACS Midwest Regional Meeting, Manhattan, KS, November, 1990

15. "Synthesis of Some New Functionalized Tetradentate Ligands and their Complexes with Rh(III) and Characterization of Some Ni(II) and Cu(II) Complexes with these Ligands", T. Kilcoin and EOS.

16. "Synthesis, Characterization, and Structural Studies of Au(III) and Rh(III) Complexes of 2-

Picolylamine Related Ligands and their Bifunctional Counterparts", N. Weerasinghe and EOS.

Society of Nuclear Medicine, Annual Meeting, Cincinnati, OH, June, 1991

17. "Radiolabeling Proteins Using Phenylenediamine Schiff Base Functionalized Chelating Agents", L. Lang, A.R. Ketring and DET.

National ACS Meeting, Atlanta, GA, May, 1991

18. "Synthesis and Characterization of Some New Functionalized Complexes of Ni(II), Cu(II), and Rh(III) Metal Ions Along with Some Radiochemical Studies with the NH_2 Derivatives of the Tetradentate Ligands Used with Rh(III)", T. Kilcoin and EOS.

Missouri Valley Society of Nuclear Medicine, Section Meeting, September, 1991

19. "Development of Functionalized 2-Hydroxy Acetophenone and Tetraphenylporphyrin Ligands for use in Labelling Proteins with the Metallic Radioisotopes Pd-109 and Rh-105", P. Droege, A. Damon, D. Whitener, and EOS.

20. "Synthesis, Characterization and Radiochemical Studies of Some Pyridyl-Amine Based Complexes of Au(III), Rh(III) and Pd(II) for Possible Use as Radiotherapeutic Agents", EOS and N. Weerasinghe.

Midwest Regional ACS Meeting, Omaha, NE, November, 1991

21. "Development of Porphyrin Ligands for Potential Use in Labelling Proteins with Metallic Radioisotopes", EOS and P. Droege.

22. "Synthesis and Characterization of Some Pyridyl-Amine-Based Complexes of Au(III), Rh(III), and Pd(II) for Possible Use in Radiopharmaceuticals", EOS and N. Weerasinghe.

Present State of Knowledge in the Field

A recent book (1), edited by David M. Goldenberg, provides an excellent summary of the general status of use of radiolabeled antibodies in cancer imaging as well as their therapeutic applications. D.M. Goldenberg, A.R. Fritzberg, C.F. Meares, and others are major contributors to development of this field. The chapter by Subramanian and Meares concludes with this statement about future prospects: "Although several radiometal-labeled monoclonal antibodies have been investigated for radioimmunoimaging and a few for radiotherapy, a number of potentially useful radiometals have not been explored...A great deal of further work is needed in order to discover and develop the best ways for using radiolabeled monoclonal antibodies clinically."

Work on the radionuclides Rh-105, Au-199, Cu-67, and Pd-109 has been limited and definitely is in need of further exploration and development. Work on Y-90, Re-188, and Re-186 has received more attention by other workers and is not addressed in this work.

The ultimate goal is to develop suitable monoclonal antibody-radionuclide conjugates with appropriate decay properties ($t_{1/2}$, β energy, γ availability for imaging, etc.) to provide for radiotherapeutic treatment of a variety of tumors. The field has not nearly advanced to the point of achieving this lofty goal.

1. David M. Goldenberg, Editor, "Cancer Imaging with Radiolabeled Antibodies," Kluwer Academic Publishers, Boston, 1990.

Division of Federal Support for Overall Program

This is the only federal support for this program. E.O. Schlemper has no other federally supported research program. However, he is the P.I. on an NSF major instrument grant for x-ray diffraction equipment and maintenance (1990-93). This is a departmental instrument operated by a department crystallographer. Schlemper only supervises the operation. Silvia Jurisson has no other federal support (new faculty member this fall). She will be submitting proposals for related work on Tc-99m, Re-186, and Re-188 to federal agencies such as NSF. It will be completely independent of that proposed here. Alan Ketring is presently a Co-I on another DOE grant: DE FG02-89ER60875, "Production of Radiolabeled Monoclonal Antibody Conjugates of Photoaffinity Labeling," (25% effort and 33.3% shared credit: $65,388 to Ketring during the current year, Wynn Volkert, P.I.). He has been on 20% effort on our present funding cycle, but that is reduced to 10% for the current proposal.

DATE
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